Characterisation of the role of Fas in intestinal inflammation and cancer

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by

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Declaration

I hereby declare that this thesis is the result of my own work and has not been submitted in whole or in part elsewhere for any award. Any assistance and contribution by others to this work is duly acknowledged within the text.

Philana Fernandes

List of publications

This work has been published in the following formats:

Journal article

Philana Fernandes, Charlotte O'Donnell, Caitriona Lyons, Jonathan Keane, Tim Regan, Stephen O'Brien, Padraic Fallon, Elizabeth Brint and Aileen Houston

Intestinal Expression of Fas and Fas Ligand Is Upregulated by Bacterial Signaling through TLR4 and TLR5, with Activation of Fas Modulating Intestinal TLR-Mediated Inflammation Journal of Immunology 2014 November 5 doi: 10.4049/jimmunol.1303083

Oral Presentations

Irish Association for Cancer Research Annual Meeting, Galway, Ireland
Fernandes, P., Lyons, C., Brint, E., and Houston, A. (2014) Fas modulates intestinal TLR-mediated inflammation and suppression of Fas in colon cancer cells reduces tumour growth *in vivo*.

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Fernandes, P., Brint, E., and Houston, A. (2012) Fas (CD95) Signalling and Inflammation in Colon Cancer.

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Irish Association for Cancer Research, Annual Meeting, Galway, Ireland
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European Association for Cancer Research, Annual Meeting, Dublin, Ireland
Fernandes, P., Brint, E., and Houston, A. (2012) Fas (CD95) Signalling and Inflammation in Colon Cancer.

Keystone Symposium - Innate Immunity: Sensing the Microbes and Damage Signals, Colorado, USA.
Fernandes, P., Houston, A., and Brint, E. (2012) TLRs 4 and 5 upregulate Fas and FasL expression in intestinal cancer cells resulting in increased cytokine production.

Abstract

Background The role of Fas (CD95) and its ligand, Fas ligand (FasL/CD95L), is poorly understood in the intestine. Whilst Fas is best studies in terms of its function in apoptosis, recent studies suggest that Fas ligation may mediate additional, non-apoptotic functions such as inflammation. Toll like Receptors (TLRs) play an important role in mediating inflammation and homeostasis in the intestine. Recent studies have shown that a level of crosstalk exists between the Fas and TLR signalling pathways but this has not yet been investigated in the intestine. **Aim** The aim of this study was to evaluate potential cross-talk between TLRs and Fas/FasL system in intestinal cancer cells. **Results** Treatment with TLR4 and TLR5 ligands, but not ligands for TLR2 and TLR9 increased the expression of Fas and FasL in intestinal cancer cells *in vitro*. Consistent with this, expression of Fas and FasL was reduced in the distal colon tissue from germ-free (GF), TLR4 and TLR5 knock-out (KO) mice but was unchanged in TLR2KO tissue, suggesting that intestinal cancer cells display a degree of specificity in their ability to upregulate Fas and FasL expression in response to TLR ligation. Expression of both Fas and FasL was significantly reduced in TRIF KO tissue, indicating that signalling via TRIF by TLR4 and TLR5 agonists may be responsible for the induction of Fas and FasL expression in intestinal cancer cells. In addition, modulating Fas signalling using agonistic anti-Fas augmented TLR4 and TLR5-mediated tumour necrosis factor alpha (TNFα) and interleukin 8 (IL)-8 production by intestinal cancer cells, suggesting crosstalk occurs between these receptors in these cells. Furthermore, suppression of Fas in intestinal cancer cells reduced the ability of the intestinal pathogens, *Salmonella typhimurium* and *Listeria monocytogenes* to induce the expression of IL-8, suggesting that Fas signalling may play a role in intestinal host defence against pathogens. Inflammation is known to be important in colon tumourigenesis and Fas signalling on intestinal cancer cells has been shown to result in the production of inflammatory mediators. Fas-mediated signalling may therefore play a role in colon cancer development. Suppression of tumour-derived Fas by 85% led to a reduction in the tumour volume and changes in tumour infiltrating macrophages and neutrophils. TLR4 signalling has been shown to play a role in colon cancer via the recruitment and activation of alternatively activated

immune cells. Given the crosstalk seen between Fas and TLR4 signalling in intestinal cancer cells *in vitro*, suppressing Fas signalling may enhance the efficacy of TLR4 antagonism *in vivo*. TLR4 antagonism resulted in smaller tumours with fewer infiltrating neutrophils. Whilst Fas downregulation did not significantly augment the ability of TLR4 antagonism to reduce the final tumour volume, Fas suppression may augment the anti-tumour effects of TLR4 antagonism as neutrophil infiltration was further reduced upon combinatorial treatment. **Conclusion** Together, this study demonstrates evidence of a new role for Fas in the intestinal immune response and that manipulating Fas signalling has potential anti-tumour benefit.

Success is failure turned inside out--
The silver tint of the clouds of doubt,
And you never can tell how close you are,
It may be near when it seems so far,
So stick to the fight when you're hardest hit--
It's when things seem worst that you must not quit.

- Author unknown –

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I would also like to thank all other members of the Departments of Medicine and Pathology, (Amruta, your exceptional cooking abilities have not gone unnoticed!) It's been a joy to know each and every one of you and, don't you worry, I'll be back (if only for Bernie's pavlova and Liam's chilli chocolate brownies!).

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I wish to dedicate this thesis to my parents, dearest Dada and Mama, my inspiration in all my endeavours. You've ignited a passion for knowledge in me that I hope will never be extinguished and your support and love has made me into the person I am today. I hope I make you proud.

Abbreviations

AOM azoxymethane

ALPS Autoimmune lymphoproliferative syndrome

APC antigen presenting cells

ARG 1 arginase 1

β-actin beta-actin

BBB blood brain barrier

BSA bovine serum albumin

BMT bone marrow therapy

CAC colitis associated cancer

CCL C-C chemokine ligand

CCR C-C chemokine receptor

CD Crohn's disease

cDNA complementary DNA

c-FLIP cellular FLICE-like inhibitory protein

CLR C-type lectin receptor

CNS central nervous system

COX cyclooxygenase

CRC colorectal cancer

CTL cytotoxic T lymphocytes

CXCL C-X-C chemokine ligand

CXCR C-X-C chemokine receptor

DAMP danger associated molecular pattern

DAPI 4',6-diamidino-2-phenylindole

DC dendritic cells

DISC death inducing signalling complex

DSS dextran sodium sulphate

EDTA ethylenediaminetetraacetic acid

ELISA enzyme-linked immunosorbent assay

EPEC enteropathogenic *Escherichia coli*

ERK extracellular signal-regulated kinases

FADD fas associated death domain

FCS foetal calf serum

GF germ free

GVHD graft versus host disease

H&E hematoxylin and eosin

HRP horseradish peroxidase

HSP heat shock protein

IAP inhibitor of apoptosis

IBD inflammatory bowel disease

IEC intestinal epithelial cell

IFN interferon

IL interleukin

iNOS inducible nitric oxide synthase

IP immunoprecipitation

i.p Intraperitoneal

IRAKm interleukin-1 receptor-associated kinase monocytes/macrophages

IRF interferon regulatory factor

JNK c-Jun N-terminal kinases

KO knockout

LBP LPS binding protein

LPS Lipopolysaccharide

mAB monoclonal antibody

MAP mitogen activated protein

M-CSF monocyte macrophage colony stimulating factor

MCP-1 Monocyte chemoattractant protein-1

MDSC myeloid derived suppressor cells

MIP3α macrophage inflammatory protein-3 alpha

MMP matrix metalloprotease

mRNA messenger RNA

MyD88 myeloid differentiation primary response gene

NF-κB nuclear factor kappa b

NGS normal goat serum

NK natural killer

NLR nucleotide binding oligomerisation domain receptor

ODN oligodeoxynucleotide

PAMP pathogen associated molecular pattern

PBS phosphate buffered saline

PGN peptidoglycan

PMA phorbol 12-myristate 13-acetate

PMN polymorphonuclear leukocytes

PPAR-γ peroxisome proliferator-activated receptor gamma

PRR pathogen recognition receptor

RA rheumatoid arthritis

RHIM rip homotypic interaction motif

RIPA radio-immunoprecipitation assay

RLR RIG-I-like receptor

RNA Ribonucleic acid

ROS reactive oxygen species

RT room temperature

RT enzyme reverse transcriptase

s.c. subcutaneous

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SEM standard error of the mean

shRNA short hairpin RNA

SIGIRR Single Ig IL-1-related receptor

SLE systemic lupus erthymatosis

T3SS type III secretion system

TAM Tumour associated macrophage

TAN tumour associated neutrophil

TBS Tris Buffered Saline

Th T helper

TIR Toll/interleukin-1 (IL-1) receptor domain

TIRRAP TIR-domain containing adapter protein

TLR toll-like receptor

TNF-α tumour necrosis factor alpha

TOLLIP toll interacting protein

TRAIL TNF-related apoptosis-inducing ligand

Tregs T regulatory cells

TRIF TIR-domain-containing adapter-inducing interferon-β

UC ulcerative colitis

WT wild type

qRT-PCR quantitative real time polymerase chain reaction

1. General introduction

1.1 The colon

The colon is a muscular tube that connects the small intestine to the rectum and its primary function is to absorb water, vitamins and minerals from food matter. The colonic mucosa, composed of a single layer of columnar epithelial cells, separates the luminal contents from the underlying vascular mucosal tissue. The mucosa is invaginated to provide increased surface area for transport and microvilli on the surface of the epithelial layer serve to further increase surface area. The mucosa also contains several specialised cells, such as the goblet, Paneth and entero endocrine (EC) cells, which secrete mucus, antibacterial molecules and peptide hormones, respectively [1, 2]. The layer of loose connective tissue beneath the mucosa is known as the lamina propria and is home to cells of the innate and the adaptive immune system such as macrophages, dendritic cells as well as B and T lymphocytes. The lamina propria is attached to the muscularis mucosa which is an innervated thin layer of outer longitudinal and inner circular smooth muscles that lends structural support to the intestine.

The intestinal lumen, in turn, is colonised by approximately 10^{14} resident commensal microbes which enable fermentation of otherwise non-digestible dietary polysaccharides thus providing nutrients and energy [3]. These bacteria also synthesize essential metabolites for the host such as vitamin K, as well as regulating important physiological functions of the host, such as those related to energy expenditure, satiety and glucose homeostasis [4-6]. Furthermore, the presence of large healthy populations of commensal bacteria prevents the emergence and proliferation of potentially harmful invading pathogens by outcompeting them for space and nutrients. Studies using germ-free (GF) mice have shown that the gut microbiota also plays an important role in the development of the gastrointestinal immune system [7]. GF mice have decreased antibody-producing lymphocytes as well as defects in the differentiation of T cell subsets and in the development of isolated lymphoid follicles, all of which culminates in a delayed immune response following antigenic challenge, relative to conventional animals [6-8].

1.1.1 Inflammation in the colon

The gut epithelium provides a structural barrier to both commensals and pathogenic bacteria and secretes a protective coat of mucus that contains antimicrobial peptides, including defensins and leukocyte protease inhibitors [9] that restrict microbial translocation. When this barrier is breached through injury or infection, or when microorganisms undergo epithelial cell transcytosis or translocation via M cells or dendritic cells (DCs), an array of subepithelial innate defence cells, especially resident mucosal macrophages, are poised for response. Located strategically in the subepithelial lamina propria, gut macrophages rapidly produce inflammatory mediators such as transforming growth factor-β (TGF-β), interleukin (IL)-8 and tumour necrosis factor alpha (TNFα) to recruit monocytes and other leukocyte populations to help contain the infection. This exerts a domino effect leading to sequential release of lipid mediators, cytokines, and chemokines that drive recruitment and activation of additional inflammatory cells. Microbes and/or their products are transported by dendritic cells via the lymphatics to draining lymph nodes where they induce an adaptive immune response. Under normal circumstances, the pathogen is successfully eliminated and inflammation is resolved. Failure to regulate and therefore resolve the normally protective cell-mediated immune response in the intestinal and/or colonic mucosa results in the sustained activation of the mucosal immune system and inflammation.

1.1.2 Chronic inflammation and inflammatory bowel disease (IBD)

Inflammatory bowel disease (IBD) includes Crohn's disease (CD) and ulcerative colitis (UC). Both are severe chronic inflammatory disorders, together affecting approximately 0.2% of the human population [10]. UC is an inflammation of the mucosa of the colon and rectum, and is characterised by ulcers and abscesses primarily confined to the mucosal and, to a lesser degree, the sub mucosal compartments [11]. Crohn's disease, by contrast, causes inflammation of the full thickness of the bowel wall and may involve any part of the digestive tract. Both conditions usually involve severe diarrhoea, pain, fatigue and weight loss [12].

Despite intense study in the last ten years, the aetiology and pathogenesis of these diseases remains unclear.

Studies investigating IBD have implicated immune, environmental and genetic factors in the pathogenesis of IBD, with a combination of these factors resulting in the induction of inflammation, followed by the development of mucosal lesions [13]. For instance, disruption of T lymphocyte regulatory functions and impairment of the mucosal immune response to normal bacterial flora has been shown to play a crucial role in the pathogenesis of chronic intestinal inflammation [14, 15]. It has also been suggested that a loss of regulatory mechanisms and a breakdown of oral tolerance to luminal antigens further contributes to the pathology seen in IBD [14, 16]. In support of this, IBD patients demonstrate abnormal T-cell responsiveness against indigenous microbiota suggesting that commensals may initiate and/or perpetuate the intestinal inflammation [17]. Indeed, recent genome-wide association studies have confirmed that microbial components play a role in the development of Crohn's disease and UC, with many patients displaying mutations in genes encoding recognition, processing and killing of microorganisms and the regulation of immune processes in the colon [18].

1.1.3 Cancer in the Gastrointestinal (GI) tract

The GI tract has a higher incidence of cancer development and cancer-related mortality than any other organ system in the body [19]. The development of colorectal cancer (CRC) can be viewed as an ordered process in which three main phases are identified: initiation, promotion and progression, reflective of the genetic alterations which drive the progressive transformation of normal healthy cells into their malignant counterparts. The inactivation of a variety of tumour suppressor genes with the simultaneous activation of oncogenes gives a selective growth advantage to intestinal epithelial cells that are subsequently no longer bound by the normal cell cycle checkpoints and restraints. Thus the transformation from normal healthy colonic epithelia to adenomatous polyp occurs. The process from adenoma to carcinoma usually takes decades and the transition from carcinoma to metastatic CRC takes an additional 2–3 years [20]. A schematic of the morphological changes

that occur in CRC along with the genetic alterations thought to be involved in neoplastic progression is shown in Figure 1.1.3.1.

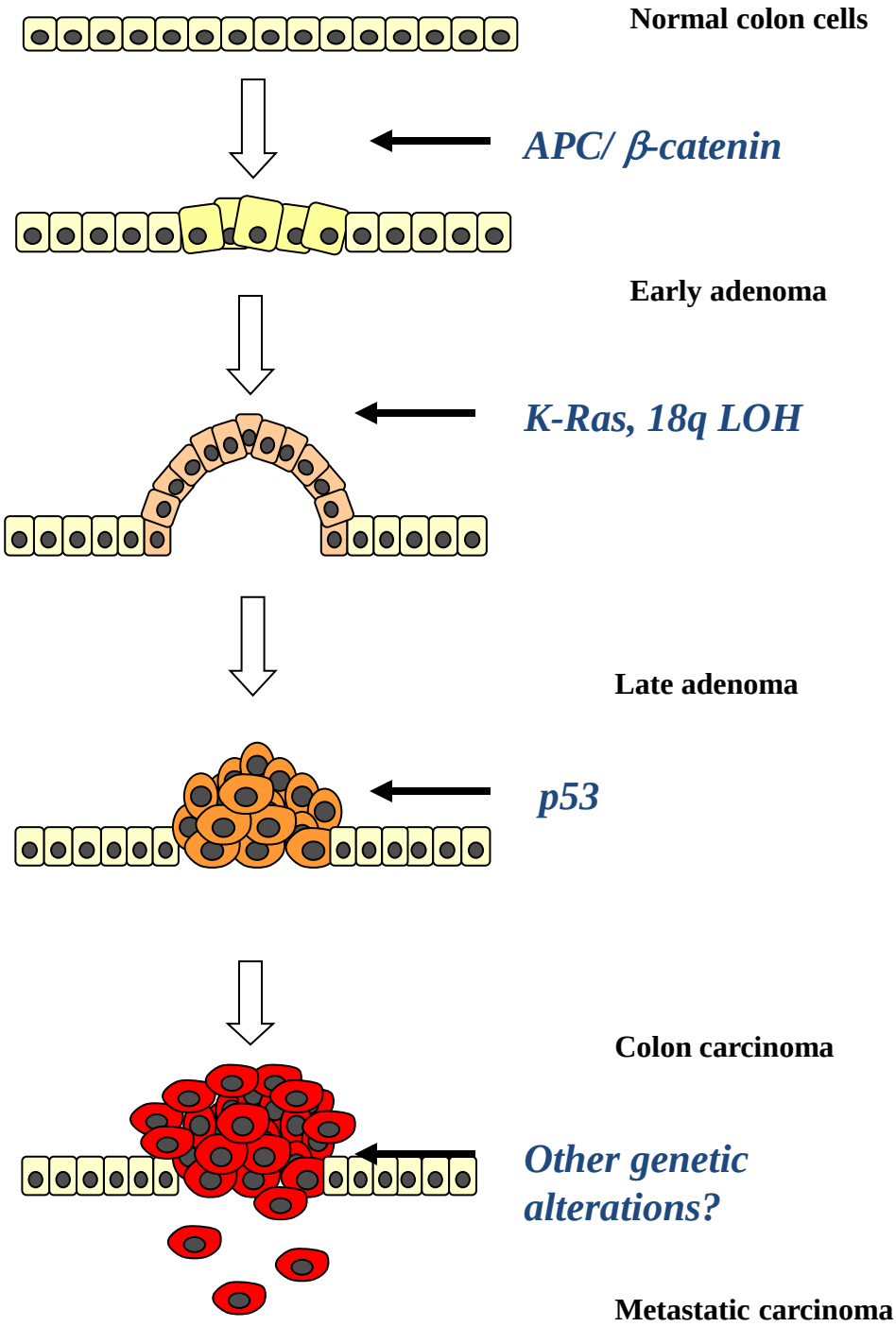


Figure 1.1.3.1. Sequential genetic alterations, corresponding with well-defined morphologic changes, in the development of colon cancer. Loss of APC occurs early in the development of adenomas, while progression from early to late adenoma is associated with mutation of Ras and LOH of chromosome 18q. Inactivation of p53 favours the progression to a malignant carcinoma.

Over the years, numerous investigations have uncovered several critical genes and pathways that are important in the initiation and progression of CRC. These include mutations in the Wingless (Wnt), Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signalling pathways, transforming growth factor beta (TGF-β), p53 and DNA mismatch-repair pathways [21]. Together these mutations culminate in the acquisition of what are referred to as the hallmarks of cancer - sustained proliferative signalling, evasion of growth suppressors, resistance to cell death, replicative immortality, induction of angiogenesis, and activation of invasion and metastasis [22]. Underlying these hallmarks are genomic instability and inflammation [23]. In addition, IBD patients have an increased risk of colorectal cancer, estimated to be between 4-20 fold relative to non-sufferers, with the risk being directly related to the duration and anatomic extent of the inflammation [24]. Furthermore, the mortality in patients diagnosed with colorectal cancer in the setting of IBD is higher than for sporadic colorectal cancer [25], suggesting the inflammatory processes characteristic of these diseases predisposes to aggressive neoplasia.

1.2 Fas (CD95) and Fas ligand (FasL/CD95L)

1.2.1 Fas (CD95)

Fas, also known as CD95, is a membrane receptor belonging to the tumour necrosis factor (TNF) receptor superfamily. It is a 45-52 kDa, highly glycosylated type 1 membrane protein with six antiparallel, amphipathic ∝-helices arranged in a fold [26], The Fas receptor has a tertiary protein structure common to all members of the TNF receptor (TNFR) superfamily (Figure 1.2.1.1a). All members have several cysteine-rich motifs (CRD) in the extracellular N-terminal domain. These motifs fold independently to the rest of the protein, and are stabilised by extensive intrachain disulphide bonding. Receptor-ligand interactions are believed to occur via these CRDs [27]. In addition, the Fas receptor and TNFR 1 share significant homology within their intracellular C terminal domains, both containing an 80-amino acid region called the death domain (DD). Fas has been shown to be predominately located at the cell surface, where it pre-associates into signalling incompetent, homodimers [28].

The human Fas gene is a single copy gene that has been mapped to chromosome 10q23 and consists of nine exons (25 bp to >1.44 kb) that are separated by eight introns (152 bp to - 12 kb), spanning 25 kb [29]. Fas is constitutively expressed on a wide variety of cell types, and is particularly abundant on T and B cells, monocytes, neutrophils, eosinophils, macrophages, epithelial and endothelial cells [30]. It is also constitutively expressed by intestinal epithelial cells. In addition, Fas expression can also be induced in some cells types in an NF-κB- and p53- dependant manner [31, 32].

1.2.2 Fas Ligand (CD95 ligand)

The cognate ligand for the Fas receptor, is Fas ligand (FasL/CD95L). FasL is synthesised as a 281 amino acid type II trans-membrane protein (Figure 1.2.1.1b). The C terminus contains the specific receptor binding domain which mediates the selective binding to the cysteine rich CRD domains of the Fas receptor. The intracellular N terminal domain of FasL contains highly conserved proline residues as well as several potential tyrosine phosphorylation sites which are likely to influence FasL protein sorting to secretory lysosomes [33].

The human FasL gene was mapped on chromosome 1q23 and consists of ~8.0 kb split into four exons [34]. NF-κB, Sp1 and EGR response elements have all been identified in the FasL promoter region [35] [36, 37]. In contrast to the ubiquitous expression of Fas, the expression of FasL is much more limited with only a few cell types constitutively expressing appreciable levels of FasL such as natural killer (NK) cells and cytotoxic T lymphocytes. FasL is also constitutively expressed in some tissues such as the eye, the testis and in some neurons where it is thought to mediate immune privilege [38, 39]. The expression of FasL is inducible in some cells such as CD4+ T helper 1 cells with expression requiring activation with antigen [40] [41]. The expression of FasL can also be induced upon cellular stress in some cells and this is thought to be dependent on JNK activation [42].

In addition to their membrane bound versions, both Fas and FasL exist as soluble proteins, sFas and sFasL, respectively [43, 44]. Although the functions of these soluble counterparts is still unclear, there is evidence to suggest that both sFas and sFasL can act as decoys, binding to either membrane bound FasL or Fas, respectively, inhibiting Fas-mediated signalling [45]. However, sFasL has also been shown to induce apoptosis on Fas bearing cells, although the biological activity of sFasL is thought to be much lower than that of mFasL [46].

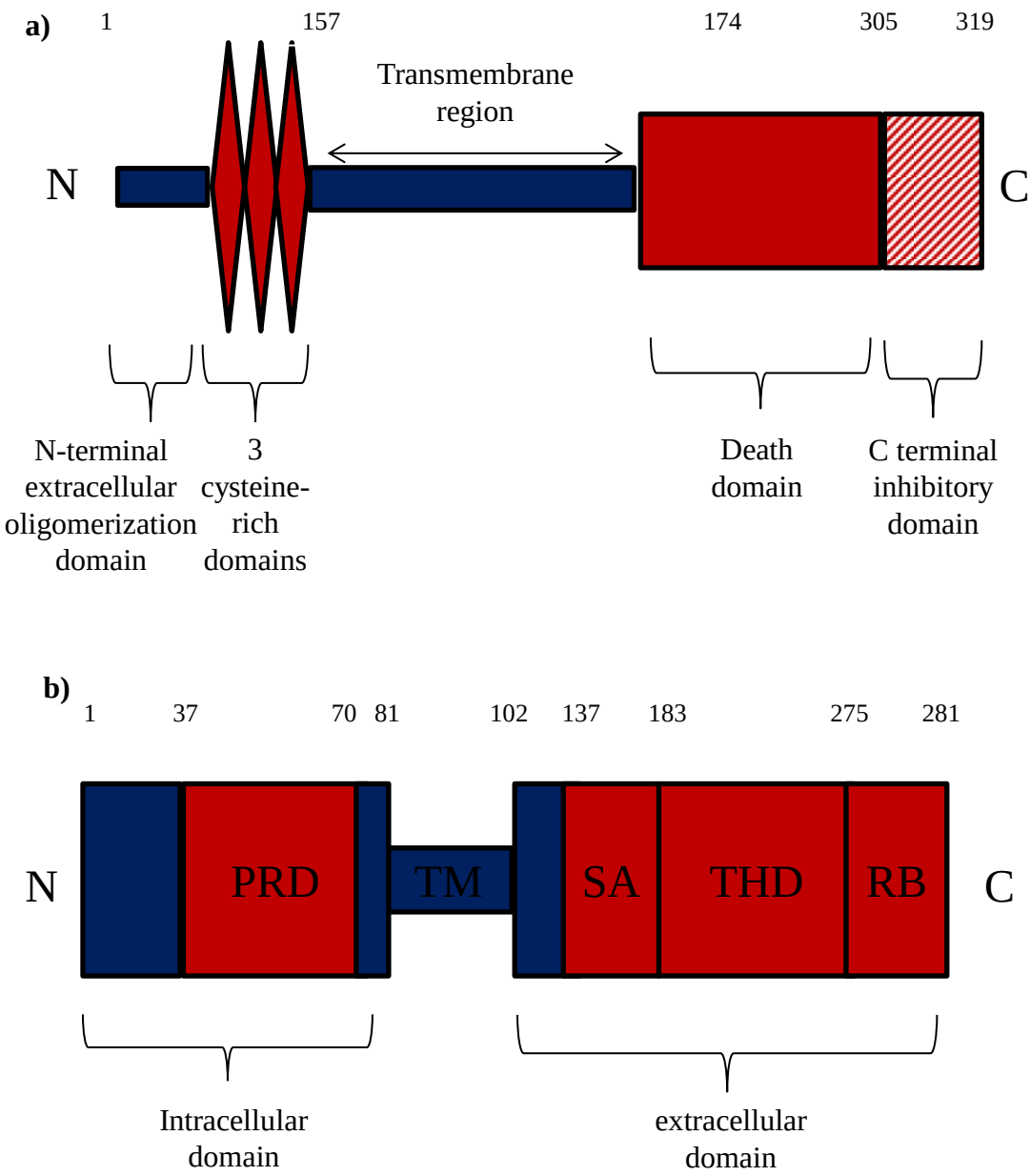


Figure 1.2.1.1 The structure of the Fas and FasL proteins

a) The mature human Fas protein consists of 319 amino acids. The N-terminal extracellular oligomerization domain is responsible for the FasL-independent oligomerization of the receptor. There are three cysteine-rich domains. A cytoplasmic death domain is crucial for apoptotic signalling. The last 15 amino acids constitute a C-terminal inhibitory domain.

b) FasL is a highly conserved transmembrane protein. The extracellular domain contains the self-assembly (SA) and receptor binding (RB) domain; whilst the intracellular domain contains motifs important for signalling including the proline-rich domain (PRD).

1.2.3 Fas-mediated apoptosis

Fas activation requires the association of trimeric FasL–Fas ($FasL_3$–Fas_3) complexes [47] which reorganises and aggregates signalling-incompetent pre-assembled Fas complexes, leading to the formation of a high stability, 'supramolecular', signalling-competent complex [48]. Formation of the supramolecule along with other intermediate steps such as actin reorganization [49], and association with membrane rafts [50], allows for the recruitment of the adaptor molecule, Fas-associated death domain (FADD), via DD–DD interactions. FADD also contains a death effector domain (DED), which in turn recruits pro-caspase-8 (FLICE) and/or pro-caspase-10 to the Fas receptor via DED-DED interactions. The resulting multimeric protein complex is called the death-inducing signalling complex (DISC), and forms within seconds of receptor engagement [51] (Figure 1.2.3.1). At the DISC, the initiator caspase, pro-caspase-8 (and/or -10), is activated. Caspases are cysteine proteases that cleave their substrates at aspartic acid residues [52]. Synthesized as inactive zymogens called pro-caspases, initiator caspases (e.g. caspases-8 and -9) require dimerization for activation [53]. Executioner caspases (Caspases-3, -6 and -7) are produced as inactive pro-caspase dimers that must be cleaved by initiator caspases. In type I cells, sufficient levels of caspase-8 are activated at the DISC, leading to the cleavage and activation of executioner caspases. [54]. Once activated, a single executioner caspase can cleave and activate other executioner caspases, leading to an

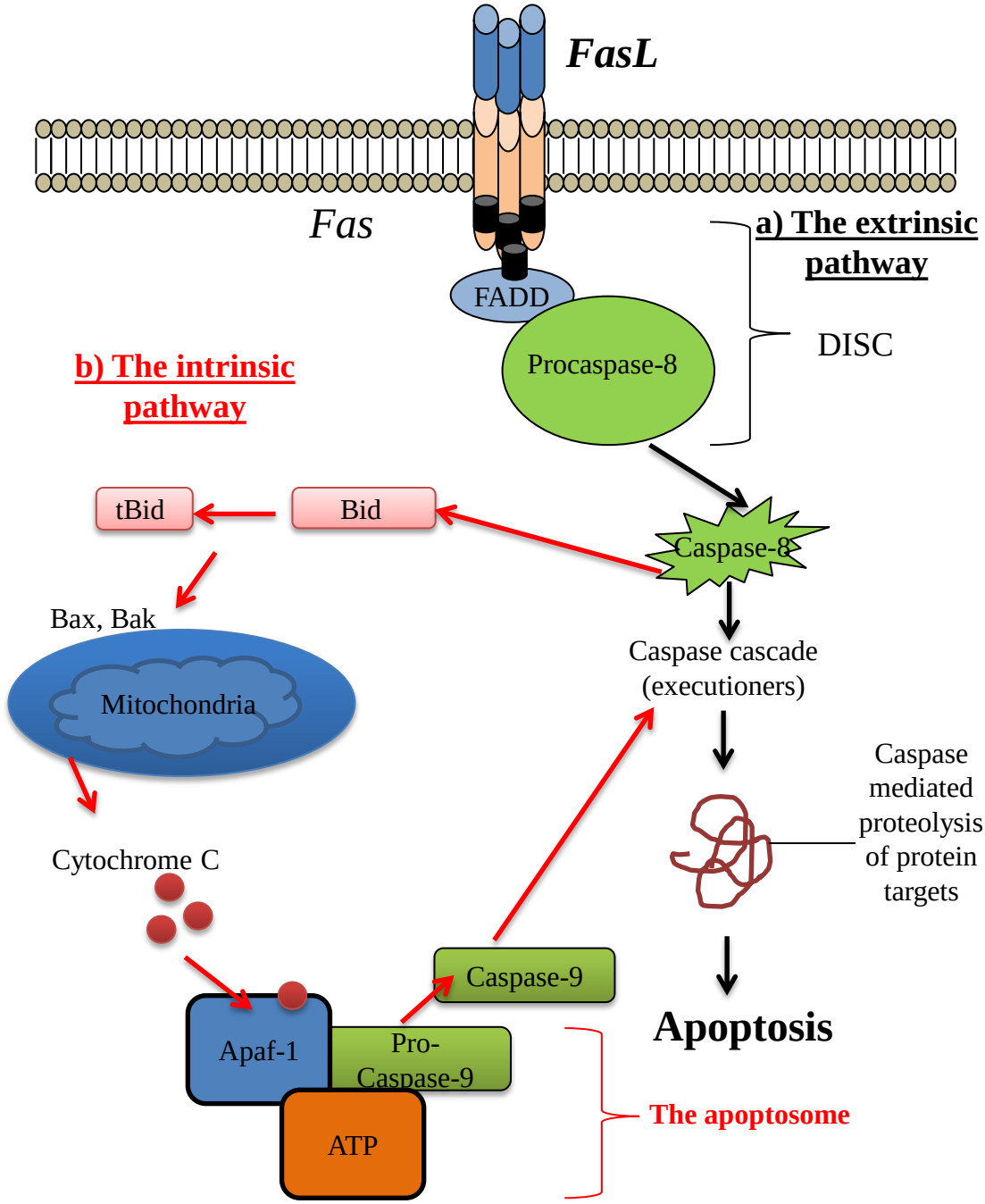


Figure 1.2.3.1 The extrinsic and intrinsic pathways of apoptosis

a) The extrinsic pathway

When cell surface Fas engages with Fas ligand, several proteins are recruited to the intracellular death domain of Fas to form the death-inducing signalling complex (DISC). This results in the proteolytic processing and autoactivation of pro-caspase 8. In type I cells, this activated 'initiator' caspase, then activates a number of intracellular executioner caspases. This caspase cascade leads to the caspase-mediated proteolysis of specific protein targets. **b) The intrinsic pathway**

In type II cells, caspase-8 mediated cleavage of Bid to tBid leads to its translocation to the mitochondria, eventually leading to the release of caspase C from the mitochondrial intermembrane space. This leads to the formation of the apoptosome within which pro-caspase 9 is activated. Caspase 9 subsequently activates the initiator caspases resulting in cell death.

accelerated feedback loop of caspase activation (Figure 1.2.3.1a).

In other cells, an insufficient amount of caspase 8 is activated at the DISC to allow for the direct induction of cell death. In these type II cells, caspase-8 must first activate the mitochondrial or 'intrinsic' apoptotic pathway [55]. This mitochondrial or 'intrinsic' apoptotic pathway is activated by caspase-8-mediated cleavage of the Bcl-2 family member, Bid. Truncated Bid translocates to the mitochondria, where it can induce both the oligomerization of pro-apoptotic Bax and/or Bak in the membrane and the release of pro-apoptotic molecules, including cytochrome C, from the mitochondrial intermembrane space. Cytochrome C can then associate with the scaffolding protein Apaf-1, dATP and pro-caspase-9 to form a high-molecular mass complex called the Apoptosome [56]. Within the Apoptosome, pro-caspase-9 is activated. Caspase-9 then activates caspase-3, resulting in cell death [56] (Figure 1.2.3.1b).

Type I and II cells differ in their content of intracellular inhibitor of apoptosis proteins (IAPs), which block executioner caspase function unless suppressed by proteins released from the mitochondria [57]. Members of the Bcl-2 family, which includes both positive regulators such as Bax, Bak, and Bid and anti-apoptotic molecules such as Bcl-2 and Bcl-x_L, also vary between type I and type II cells [24]. Expression of either Bcl-2 or Bcl-x_L renders Type II cells resistant to Fas-mediated apoptosis. Type I cells however, cannot overcome the production of the large amounts of caspase-8 produced at the DISC, and are therefore not protected from Fas-mediated apoptosis even by the expression of very high levels of Bcl-2 or Bcl-x_L [58].

Both the intrinsic and extrinsic pathways of apoptosis culminate in the activation of the end stage caspases which ultimately cause the morphological and biochemical changes seen in apoptotic cells [59]. These include degradation of chromosomal DNA, chromatin condensation, cytoskeletal reorganisation, and finally phosphatidylserine externalization which allows for phagocytic recognition and uptake of the apoptotic cells [60].

1.2.4 The physiological and pathological functions of the Fas/FasL apoptotic signalling pathway

Characterisation of *lpr* and *gld* mice which exhibit lymphadenopathy and splenomegaly revealed mutations in Fas and FasL, respectively [61, 62], with the defects in these mice mutants providing some of the first evidence for a role for Fas/FasL signalling in the immune system. These mutations are loss of function mutations, resulting in impaired Fas-mediated apoptosis. Activation-induced cell death (AICD) is the primary homeostatic mechanism used by the immune system to control T cell responses in the peripheral lymphoid organs, restoring normal cell number following T cell expansion in response to infection [63]. Fas-mediated apoptosis has been shown to play a critical role in AICD. Fas-mediated apoptosis is also critical in the clonal deletion of auto reactive T cells [64]. Furthermore, following target cell recognition, FasL is upregulated on cytotoxic CD4+ T cells, which subsequently binds to Fas on the target cell thus executing the specific killing of virally infected or transformed cells [65].

The Fas/FasL system also plays an important role in immune privilege with some tissues, such as the eye and the testes constitutively expressing Fas ligand [66-68]. Constitutive expression of FasL in such tissues has been shown to trigger apoptosis of activated Fas-bearing inflammatory cells entering these sites, thereby protecting these tissues from a potentially disastrous inflammatory immune response [38].

Whereas the Fas/FasL apoptotic system normally contributes to the killing of virally infected, damaged or excess cells, abnormally increased levels of Fas-mediated apoptosis has been implicated as a cause of certain immunopathological disorders. For instance, Fas-mediated apoptosis has been implicated, alongside perforin, in the destruction of insulin-producing cells and the development of diabetes [69-71]. Insufficient levels of Fas-mediated apoptosis have also been implicated as a cause of disease with the Fas/FasL system being involved in the pathogenesis of autoimmune lymphoproliferative syndrome (ALPS). A rare disorder of disrupted lymphocyte homeostasis, clinical manifestations of ALPS vary, but typically include abnormal enlargement of organs and lymph nodes within the first two years of life. Patients also have an increased number of double-negative T cells (CD4-/CD8-) [21]. Since

these features are shared with *lpr* and *gld* mice, mutations in either Fas or FasL genes were suspected to play a role in the development of ALPS, with subsequent studies confirming this. [16, 72-74]. Mapping these mutations to the FasL and Fas genes suggests that the resultant mutant transcripts may either be unable to bind Fas or transduce the apoptotic signal. Systemic lupus erythematosus (SLE) is an autoimmune disease that can affect different organs including skin, heart, lungs, blood vessels, liver, kidneys, joints and the nervous system. SLE is characterised by malregulation of T and B cells causing the production of excessive auto-antibodies and the formation of immune complexes against nuclear antigens [75, 76]. The elimination of auto reactive T or B cells is impaired in SLE patients [17, 77], suggesting that the Fas/FasL signalling system may also play a role in this disease. Evidence suggests that an single nucleotide polymorphism in the enhancer region of the Fas gene promoter prevents the binding of the SP-1 transcription factor, which in turn leads to diminished Fas expression, which may contribute to the reduced ability of SLE patients to clear auto reactive T and B cells [13, 78-80].

1.3 The non-apoptotic functions of Fas

Although most extensively studied for its role as a mediator of cell death, Fas has been found to mediate a variety of non-apoptotic functions. These include proliferation, migration and inflammation (Figure 1.3.1).

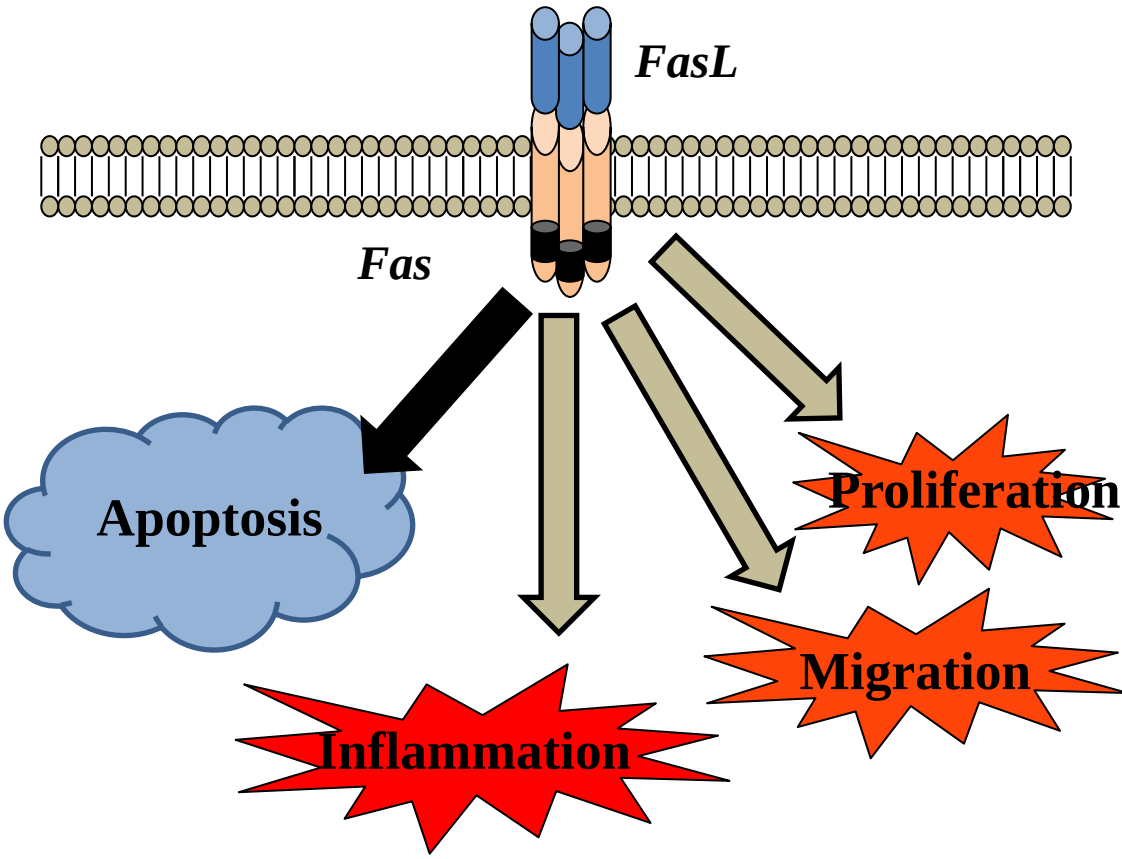


Figure 1.3.1 Signals emanating from Fas-ligation
Upon FasL ligation, the best characterised response is apoptosis in the Fas-bearing cell. However ligation of Fas has also been shown to lead to a variety of other cellular consequences including inflammation, migration and proliferation.

1.3.1 Proliferation

One of the first examples of a non-apoptotic function for Fas was the discovery that Fas could stimulate the proliferation of anti-CD3-activated human peripheral blood T cells. Activation of T cells requires two main signals with a lack of secondary co-stimulation resulting in Fas-mediated apoptotic cell death. However, despite a lack of secondary co-stimulation, T cell receptor (TCR)/CD3-stimulated memory T cells do not undergo apoptosis but instead proliferate more rapidly as a consequence of Fas ligation. This suggests that the physiological response of $CD4^+$ T cells to Fas ligation is determined by previous antigenic history and availability of co-stimulatory molecules [81]. Moreover, the outcome of Fas ligation on naïve T cells was found to depend largely on the 'dose of agonist', with a complete block of activation occurring at high doses of Fas agonist, while lower concentrations of Fas agonist had co-stimulatory activity [82]. Taken together, these data suggest that the expression levels of FasL present on antigen presenting cells (APCs) regulate the immune response, with initial low levels positively co-stimulating naïve T cells and increased FasL expression later on resulting in immune response termination by inducing apoptosis in pre-activated T cells.

A variety of non-immunological cells, including B cells and fibroblasts, have also been shown to respond to Fas stimulation with enhanced proliferation. Moreover, in the liver, although *in vivo* administration of an anti-Fas antibody caused massive hepatic apoptosis resulting in hepatic failure [83], partial hepatectomy prevented the systemic lethal effect, with Fas engagement actually accelerating the regenerative response [84]. These data indicate that liver damage stimulates a Fas-mediated protective regenerative response which may be harnessed to promote regeneration and healing.

1.3.2 Migration

Several studies have suggested that signalling through Fas can promote cell migration [85-88]. In multiple cancer cell types resistant to Fas-mediated apoptosis, urokinase plasminogen activator (uPA) was found to be required for Fas ligand-

induced motility and invasiveness [85]. After binding to its receptor, uPA cleaves plasminogen forming plasmin, which can cleave and activate matrix metalloproteinases (MMP), specifically MMP2 and MMP9, in a tyrosine kinase-dependant manner, resulting in the cleavage of further proteins such as collagen [89]. 3811}. uPA is absolutely required for intravasion [90] and it has been recognized that uPA can also induce increased motility in tumour cells [91]. *In vivo*, mice that lack FasL specifically in neutrophils and macrophages exhibit a reduced infiltration of neutrophils and macrophages to the spinal cord following transfection injury. Furthermore, FasL treatment of these cells lead to increased invasion using an *in vitro* transmigration assay, which was abolished upon pharmacological inhibition of MMP-2 and 9 [93, 94].

Tyrosine kinase activation by Fas in colorectal cancer cells has also been shown to result in the rapid actin-driven formation of cell protrusions, a process that is essential for tumour cell invasion [93]. Thus, it appears that Fas activation can promote tumour cell invasion through distinct pathways.

Fas ligation has been shown to promote neurite outgrowth and branching [95, 96] and following *in vivo* sciatic nerve injury, acceleration of nerve regeneration [95]. Fas signalling may also mediate neuronal branching [97]. Moreover, since potent immunosuppressive drugs, which completely prevent autoimmunity, fail to prevent neurological defects from developing in *lpr* mice, may suggest that neurodegeneration in the *lpr* mouse is a direct result of limited Fas expression on neuronal cells, highlighting the importance of the Fas/FasL system in the nervous system [98, 99].

1.3.3 Inflammation

Fas ligation has also been shown to induce the production of a number of proinflammatory cytokines, such as IL-8, IL-6, IL-1β and monocyte chemoattractant protein-1 (MCP-1), by a variety of non-lymphoid cells [100-102] [103, 104], [105], [106], as well as by lymphoid cells [104, 107]. Fas-induced cytokine production has been shown to involve the direct activation of NFκB, and the p38 JNK, ERK, MAPK signalling pathways. Recently, studies have suggested that myeloid differentiation primary response 88 (MyD88) may also play a role in Fas-mediated inflammation

(Figure 1.3.3.1). The Fas-induced chemokine (C-X-C motif) ligand 1 (CXCL-1) production in alveolar epithelial cells was shown to be dependent on MyD88, with Fas-induced chemokine production decreased in MyD88 deficient macrophages [108]. In addition, numerous allograft studies have demonstrated that over-expression of FasL can trigger neutrophil recruitment, ultimately leading to graft rejection [109-111].

Fas/FasL-mediated inflammation has been implicated in the pathogenesis of several diseases that have an underlying inflammatory component such as acute respiratory distress syndrome [108], cystic fibrosis [112], arthritis [104, 113] and cancer [114]. Moreover, many chronic inflammatory diseases are attenuated in mice lacking Fas or FasL [113, 115, 116]. For example, in models of pulmonary inflammation, *lpr* mice exhibit reduced cytokine secretion and neutrophil influx, together with a reduction in epithelial cell apoptosis and tissue damage [117] [118].

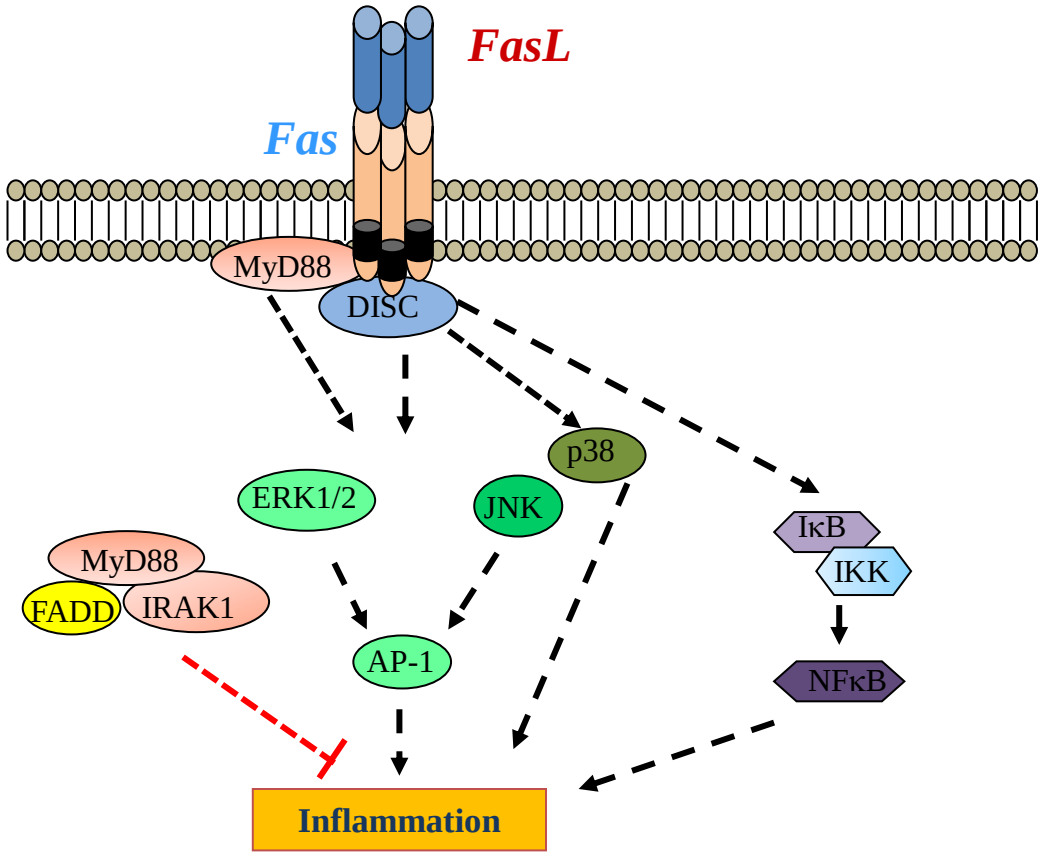


Figure 1.3.3.1. Signalling pathways involved in Fas-mediated inflammation
Ligation of Fas has been shown to promote inflammation via the activation of p38, ERK, and JNK MAPK signalling pathways as well as activation of NFκB. MyD88 may interact directly with Fas, independently of FADD, resulting in activation of the JNK and ERK MAPK signalling pathways. Alternatively, in the absence of Fas ligation, FADD may interact with MyD88 in the cytoplasm potentially reducing the stability of its interaction with IRAK1, thus limiting MyD88-dependant signalling.

1.4 Fas signalling in the intestine

Despite being constitutively expressed in the intestine, the physiological role of Fas in the colon is unclear. Fas has been shown to be constitutively expressed at the basolateral side of all epithelial cells in the colon [119, 120], while FasL is predominantly shown to be found in a few scattered lamina propria cells and Paneth cells [121]. Despite being best characterised for its role in apoptosis, there are no differences in morphology or apoptotic cell counts in the gut mucosa of *lpr* and *gld* mice relative to WT mice [122]. Indeed Fas-induced apoptosis was demonstrated to be impaired in active UC [123], suggesting that Fas signalling may actually be protective in the colon. Consistent with this, the colonic epithelium from *lpr* mice infected with *Citrobacter rodentium* (*C. rodentium)* was found to be more damaged and hyperplastic in comparison to control mice [124]. Taken together, it is therefore unlikely that the Fas/FasL system is involved in the physiological turnover of the gut epithelium.

Recent evidence suggests that Fas/FasL signalling in the intestine may play a crucial role in the host response to pathogens and in particular, pathogen clearance. Bacterial pathogens have been shown to antagonize death receptor-induced apoptosis of infected cells in the intestine [124, 125]. NleB is an effector protein of the Type III secretion system, a syringe-like apparatus that delivers bacterial effector proteins directly into the host cell cytoplasm [126]. NleB was recently shown to bind to and modify the death domain of FADD [125] and in doing so, prevent DISC formation, thereby suppressing caspase activation and subsequent cell death [124].

Infection can also induce inflammation in the host in order to promote the rapid recruitment of immune cells to combat potential pathogens and Fas ligation on intestinal cancer cells has been shown to enhance immune cell recruitment through the production of cytokines and chemokines. For example, Fas signalling increases CXCL-1 chemokine production in alveolar epithelial cells that leads to enhanced neutrophil infiltration [108]. Furthermore, *lpr* mice exhibit reduced airway epithelial cell apoptosis, cytokine secretion, neutrophil influx, and tissue damage in models of pulmonary inflammation [116]. Similar results have been obtained using small interfering RNA (siRNA) targeted against Fas [127], suggesting that Fas-

mediated inflammatory signalling may be important for effective pathogen clearance and host defence in mucosal tissues.

1.5 Fas signalling in colon cancer

Numerous studies have shown that the expression of FasL is upregulated in cancers of the gastrointestinal tract and this is thought to be an early event during neoplastic transformation [128-130]. The upregulation of FasL is associated with a poor clinical prognosis and resistance to chemotherapy [131]. By upregulating FasL, tumours are thought to be able to mount a counter attack against Fas-bearing tumour infiltrating lymphocytes, in effect potentially affording tumours the same immune privilege that is seen in areas such as the eye and the testis [132]. In support of this model, apoptosis of tumour infiltrating lymphocytes was increased within FasL-expressing areas of oesophageal, gastric and colonic tumours [133-135], while areas of human colon cancers expressing increased FasL exhibit reduced lymphocyte infiltration [136]. Moreover, subcutaneously injected colon cancer cells with reduced FasL expression exhibited reduced tumorigenicity, increased infiltration of TILs and an enhanced anti-tumour immune response *in vivo* [132, 137] providing functional evidence in favour of a role for FasL in colorectal cancer.

Signalling through Fas by FasL has also been shown to mediate migration and invasion of tumour cells, and to play an important role in tumour recurrence following treatment of colorectal liver metastases. FasL is more frequently expressed in liver metastases than in primary colon carcinomas [138] [139], with FasL signalling shown to lead to the accelerated outgrowth of micro metastases [140] [141]. These data indicate that tumour-expressed FasL may also be important in the colonization of colon cancer cells in tissues distant to the original neoplastic site such as the liver [142].

Despite extensive studies demonstrating a role for FasL in tumorigenesis, allograft studies, whereby cells were engineered to over-express FasL, demonstrated that FasL triggered extensive neutrophil recruitment and allograft destruction. These neutrophil recruitment studies indicated that by recruiting tumouricidal neutrophils, tumour-derived FasL expression may potentially mediate tumour rejection [143-

145]. A caveat to this conclusion is however necessary, since all studies used cells that were engineered to overexpress FasL, which is rarely reflective of the physiological situation.

In contrast to the wealth of investigations into the role of FasL in tumorigenesis, there are relatively few studies focusing on Fas and its role in neoplasia. The carcinogen, azoxymethane (AOM) can be used in combination with the inflammatory agent, dextran sodium sulphate (DSS) to induce colon cancer in rodent models [146]. An investigation into the potential role of Fas signalling in the intestine using the AOM/DSS mouse model demonstrated that deletion of intestinal cancer cell-expressed Fas resulted in a greater number of colonic tumours relative to mice with wild type intestinal cancer cells [147], perhaps suggesting that Fas acts as a tumour suppressor in this tissue. However, since DSS was used to induce colitis in this investigation, the ensuing inflammation may have itself promoted colon carcinogenesis and therefore effectively negated a potentially protective effect of a reduction of tumour-expressed Fas. Indeed another investigation suggested that Fas acts as a tumour promoter in the colon, with Fas deficiency leading to an increase in tumour burden relative to control mice with normal Fas expression levels [148].

Thus, the role of the Fas/FasL system in the intestine is unclear and warrants further characterisation.

1.6 Innate immunity

1.6.1 Pathogen Recognition Receptors

The innate immune system constitutes the first line of host defence against infection and so plays a crucial role in the early recognition and initiation of a proinflammatory response to invading pathogens. The innate immune response relies on recognition of evolutionarily conserved signature molecular structures on pathogens, termed pathogen-associated molecular patterns (PAMPs). PAMPs are recognised by a limited number of germ line-encoded pattern recognition receptors (PRRs). Several families of PRRs, including the membrane associated Toll-Like receptors (TLRs), the cytosolic NOD-like receptors (NLRs) and the RIG-I-like

receptors (RLRs), are known to play a crucial role in host defence (Figure 1.6.1.1). TLRs ligate and respond to PAMPS present on the cell surface or in host endosomes; NLRs recognise microbial molecules in the host cytoplasm whilst the RLRs promote a response to viral RNA strands present in the host. In addition, recent studies have suggested that human caspase 4, and the mouse homologue, caspase 11, play an important role in LPS-induced mortality [149] .

PAMPs are essential invariant functional components of microorganisms not usually present in the host cell and are therefore distinguishable from "self". Major PAMPs are microbial nucleic acids, including DNA (e.g. unmethylated CpG motifs), double-stranded RNA (dsRNA), single-stranded RNA (ssRNA), as well as lipoproteins, surface glycoproteins, and membrane components such as peptidoglycans, lipotechoic acid, lipopolysaccharide (LPS) and Flagellin. PRRs can also recognize host factors as "danger" signals when they are present in aberrant locations or in abnormal molecular complexes such as heat shock proteins (HSP), and high-mobility group box protein 1 (HMGB1), and these are known as danger associated molecular patterns (DAMPs) [150, 151].

Following PAMP or DAMP recognition, PRRs activate a multitude of intracellular signalling pathways, including the MAPK signalling pathways, as well as activating transcription factors such as NF-κB. This ultimately results in the activation of gene expression and the synthesis of a broad range of molecules, including cytokines, chemokines, cell adhesion molecules and immunoreceptors, which together orchestrate the early host response to infection [152] (Figure 3.6.1.1).

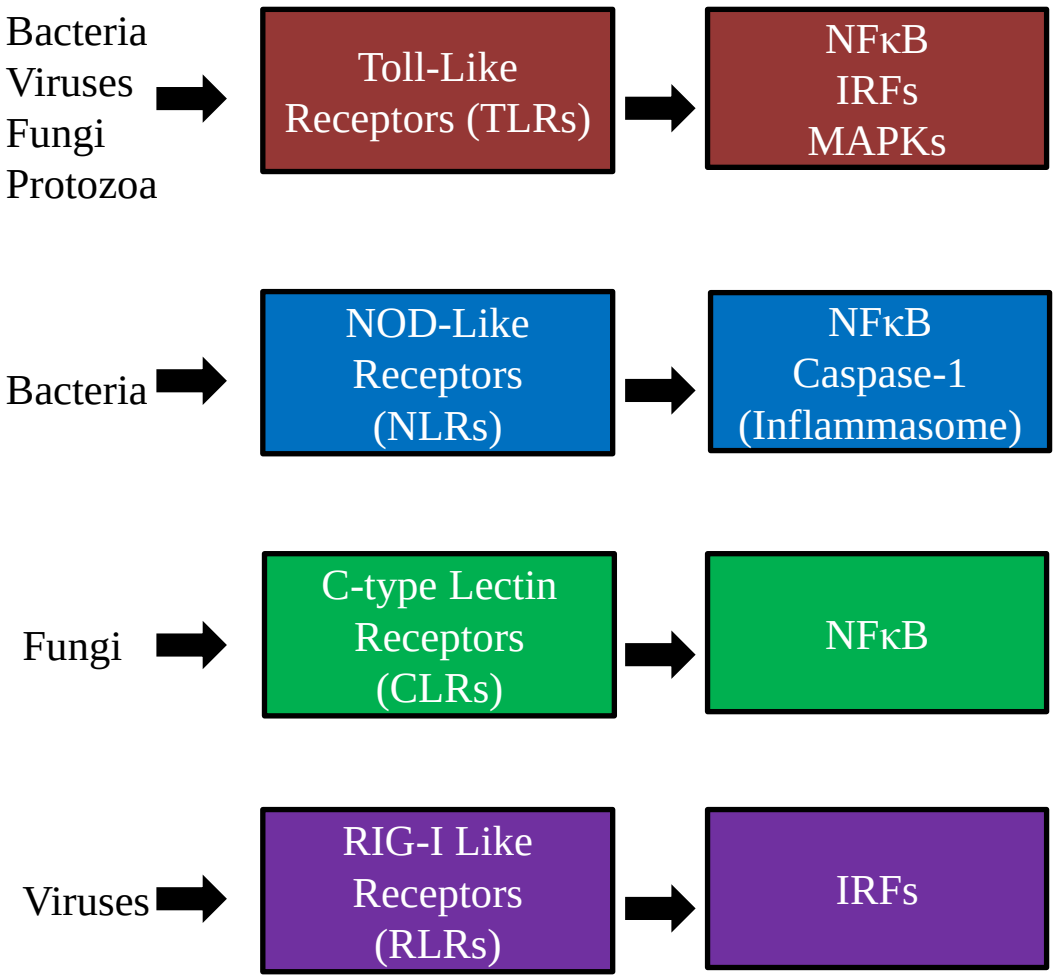


Figure 1.6.1.1 Examples of known PRRs and their function.
Members of each PRR family member specified above detect specific PAMPs from various pathogens as indicated. Upon recognition of each PAMP, the PRRs initiate a downstream signalling cascade leading to the eventual activation of transcription factors which in turn induce expression of the appropriate immune response genes.

1.6.2 The Toll-Like Receptors

There are thirteen known mammalian TLR genes, eleven of which are expressed in humans. TLRs vary in their distribution between the plasma and endosomal membranes; TLRs 1, 2, 4-6 are located at the plasma membrane whilst TLRs 3 and 7-10 are found endosomally. Cell surface TLRs mainly recognize microbial membrane components such as lipids and lipoproteins. For example, TLR4 recognizes bacterial LPS and there is evidence to suggest the existence of viral ligands for TLR4 [153]. TLR2, along with either TLR1 or TLR6, recognizes a wide variety of PAMPs including lipoproteins, peptidoglycans, lipotechoic acids and zymosan. TLR5 recognizes bacterial Flagellin. Intracellular TLRs include TLR3 and TLRs 7-10. TLR3 recognizes viral dsRNA, small interfering RNAs and self-RNAs derived from damaged cells whilst TLR7 is predominantly expressed in plasmacytoid DCs (pDCs) and recognizes ssRNA from viruses. Human TLR8 responds to viral and bacterial RNA whilst TLR9 recognizes bacterial and viral DNA that is rich in unmethylated CpG-DNA motifs. Intracellular TLR10 has recently been shown to collaborate with TLR2 to recognize ligands from *Listeria Monocytogenes* [154]. A table showing the location of TLRs and their identified ligands is shown in Table 1.

The TLRs are type I integral membrane receptors, containing an N-terminal ligand recognition domain, a single trans-membrane helix, and a C-terminal cytoplasmic signalling domain [155], known as Toll IL-1 Receptor (TIR) domains due to the fact that they share homology with the signalling domains of IL-1R family members [156]. The transmembrane domains of the TLRs each contain a typical stretch of approximately 20 uncharged, mostly hydrophobic, residues. The N-terminal ligand recognition ectodomains (ECDs) of TLRs are glycoproteins composed of 550–800 amino acid residues [155]. PAMPs bind to the receptor ECD and induce the dimerization of the TIR domains, forming a scaffold for downstream signal transducers. Cell-surface TLRs are monomeric but form active homo- or heterodimers when exposed to PAMPs.

Receptor	Location	Ligand	Ligand origin
TLR1	Cell surface	Multiple triacyl lipopeptides	Bacteria
TLR2	Cell surface	multiple glycolipids multiple lipoproteins HSP70 zymosan	Bacteria Bacteria Host Fungi
TLR3	Cell compartment	Double-stranded RNA	Viruses
TLR4	Cell surface	lipopolysaccharide heat shock proteins fibrinogen	Gram-negative bacteria Bacterial and host cells Host
TLR5	Cell surface	Flagellin	Bacteria
TLR6	Cell surface	multiple diacyl lipopeptides	Mycoplasma
TLR7	Cell compartment	Single stranded RNA	RNA Viruses
TLR8	Cell compartment	Single stranded RNA	RNA Viruses
TLR9	Cell compartment	Unmethylated CpG Olidodeoxynucleotide DNA	Bacteria, DNA Viruses
TLR10	Cell surface/cell compartment	?/*L.monocytogenes* ligand?	?/bacteria

Table 1 TLRs and their ligands
Intracellular location of each TLR with their known respective ligands and the origin of each ligand.

Heterodimerisation between TLRs or the association with co-receptors such as CD14 or CD36 increases the diversity of molecules that can be recognized by TLRs [157, 158]. Whilst the majority of TLRs traffic directly to the cell surface after their synthesis, TLRs7–9 are synthesized as stable preformed dimers [159, 160]. Irrespective of the mode of dimerization, ligand-activation of TLRs brings the sequences at the C terminus of the two ECDs into close proximity which is imperative for downstream signalling.

1.6.3 TLR signalling pathways

There are 2 main adaptor proteins that bind to TLRs and facilitate signal transduction. These are known as Myeloid Differentiation factor 88 (MyD88), and TIR-domain-containing adapter-inducing interferon-beta (TRIF). Signal transduction via MyD88 or TRIF can also be mediated by the bridging adaptor proteins, MyD88-adapter-like (MAL) or TRIF-related adaptor molecule (TRAM) respectively (Figure 1.6.3.1). MAL is a sorting adaptor that recruits MyD88 to cell surface TLRs such as TLR2 and TLR4. However, a recent study demonstrated that MAL also participates in signalling through endosomal TLRs such as TLR9 [161]. TRAM is selectively recruited to TLR4, but not TLR3, to link between TRIF and TLR4 whilst TLR3 directly interacts with TRIF.

MyD88 is a modular protein containing a death domain (DD) that is connected to a TIR domain by an intermediate domain. During MyD88-mediated TLR signal transduction, MyD88 assembles with the DD-containing IL-1R-associated kinase (IRAK) family, four of which are found in vertebrates: IRAK1, IRAK2, IRAKM (also known as IRAK3) and IRAK4. *In vitro*, the MyD88 DD forms a heterogeneous mixture of dimers and higher-order oligomers but in the presence of IRAK4, these assemble into a discrete heterocomplex, coined the Myddosome [162], the composition of which is thought to depend on the cellular context [163]. IRAK4 phosphorylates IRAK1, which is also recruited to the complex, leading to its activation. IRAK1 then dissociates from the complex and interacts with TNF receptor associated factor -6 (TRAF6). TRAF6 is the activator of the canonical NF-κB pathway (Hayden and Ghosh, 2004). TRAF6 becomes ubiquitinated at residue K63 and this modification allows TRAF6 to activate the next component in the pathway, which is most likely to be TGF-β activated kinase-1 (TAK1) (Sun et al.,

2004). TAK-1 then activates the inhibitory κB (IκB) kinase complex leading to activation of NF-κB, and upstream kinases for the p38 and JNK MAPK signalling pathways, leading to the production of a number of cytokine and chemokines (Figure 1.6.3.1).

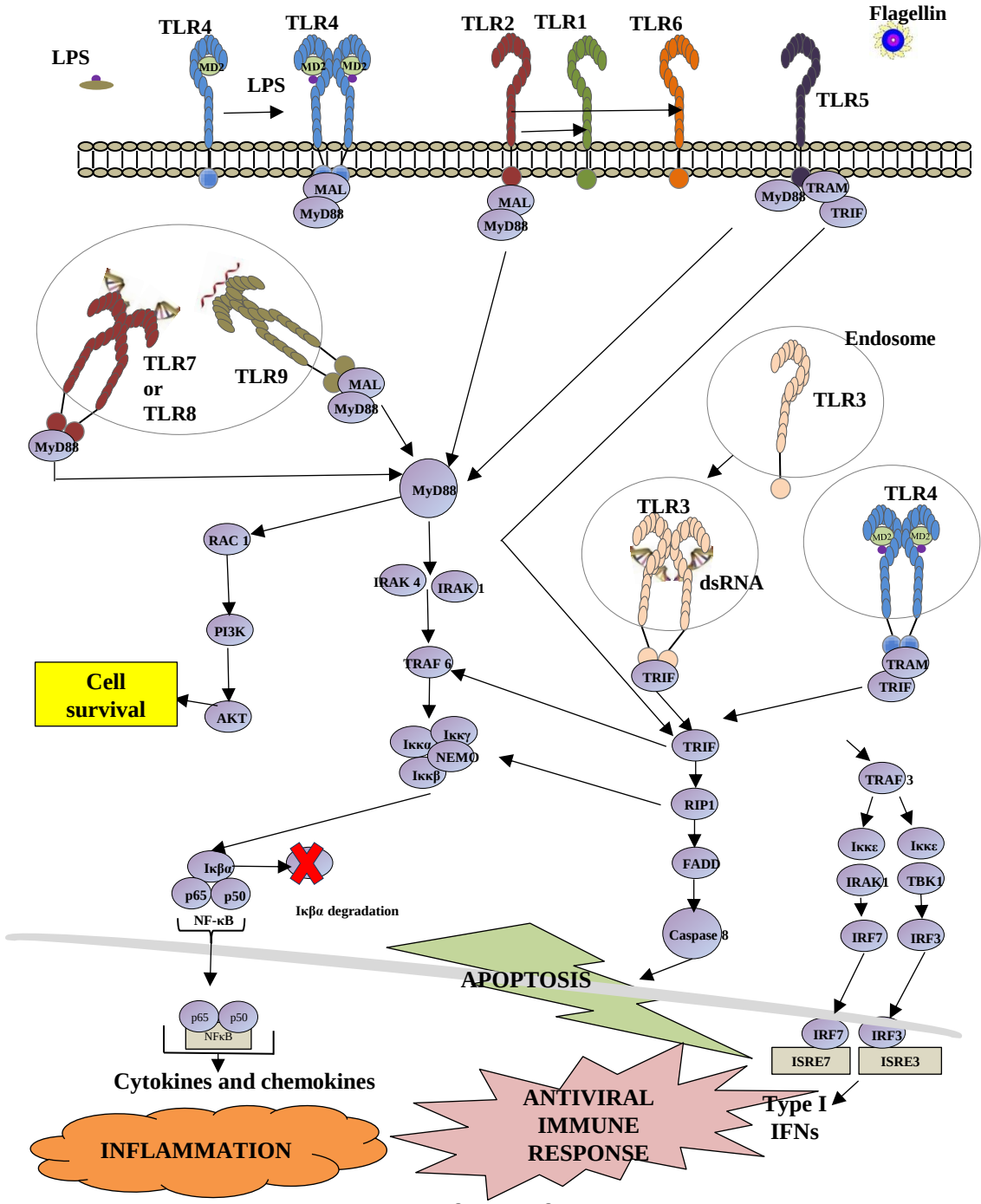


Figure 1.6.3.1 Signal transduction of TLRs following ligand recognition. Following ligand recognition, each TLR initiates a downstream signalling cascade using adaptor molecules. All TLRs signal through MyD88 with the exception of TLR3 which requires TRIF. TLR4 and TLR5 may also signal through TRIF with the using TRAM. Recruitment of MyD88 to the TLR receptor initiates downstream activation of TRAF6, which in turn, activates NF-κB resulting in the transcription of inflammatory cytokines. Alternatively, if TLR4 is trafficked to the endosome upon ligand recognition, it recruits TRAM, which recruits TRIF. TLR3 also recruits TRIF upon ligand binding. This initiates downstream activation of TRAF3 and subsequently activates the IRF transcription factors which induces production of the anti-viral interferon genes. TLR-mediated MyD88 signalling can also lead to the activation of the PI3K cell survival pathway whilst TLR-mediated TRIF signalling has been shown to activate the apoptotic pathway.

MyD88 is required by all TLRs for signal transduction except for TLR3, which uses the alternative adaptor protein, TRIF (Figure 1.6.3.1). TLR3 has an alanine rather than a proline in the TIR domain BB loop which is unique to human TLRs and gives TLR3 its specificity for TRIF-dependant ligation and signalling. Mutation of this one residue in TLR3 to proline causes a switch in adaptor protein specificity from TRIF to MyD88 resulting in attenuated IRF3-dependant signalling and enhanced NFκB activation [164]. Relative to MyD88, TRIF has a more complex multimodular structure of 712 amino acids. The α-helical N-terminal domain of TRIF is followed by a proline-rich region with binding sites for the downstream effector proteins TRAF2 and TANK-binding kinase 1 (TBK1). The TIR domain and a receptor-interacting protein (RIP) homotypic interaction motif (RHIM) domain constitute the C terminus of TRIF [165-167]. The TIR domain of TRIF binds to TLR3. It can also bind to TLR4–TRAM leading to the binding of TBK1 and TRAF3, and ultimately the activation of IRF3 and/or IRF7. This pathway results in IRF1-dependent type I interferon production via a mechanism involving the activation of IRF3. TRIF can also activate NFκB by activating TRAF6 [166].

Recently, it has been shown that TLR5 also utilises TRIF, at least in intestinal cancer cells, in a process that requires internalisation of TLR5 into endosomes [168]. However, it is likely that TRIF mediated TLR5-induced responses are associated with regulating NFκB and MAPK activation rather than IRF-3 activation and IFN-β expression in order to mediate TLR5-dependent inflammatory responses. Uniquely, TLR4 can activate both the MyD88 dependant and TRIF dependant (Myd88-independent) signalling pathways with the LPS-induced production of proinflammatory cytokines such as TNF-α or IL-6 requiring both MyD88- and TRIF-dependent pathways [169, 170]. LPS is also capable of activating MyD88-independant signalling in order to produce interferons [171].

Due to their integral role as sentinels of the innate immune response, TLR-mediated inflammation is of paramount importance in resolving infection and mediating tissue healing. In addition to playing a key role in host defence against danger, activation of TLRs has been linked to the pathogenesis of many inflammatory and autoimmune diseases including rheumatoid arthritis (RA), sepsis, systemic lupus erythematosus (SLE), inflammatory bowel disease (IBD) and type I diabetes. For example, TLR-mediated microbial recognition in the intestine is important in the development of

chronic enterocolitis [172] and aberrant that the MyD88 dependant TLR-mediated signalling is thought to be responsible for the development of the atherosclerosis [173, 174]. In addition, in patients with SLE, the normally limited numbers of auto-reactive B and T cells that are present in healthy individuals are expanded, producing large quantities of autoantibodies, directed especially against nuclear antigens such as those that act as TLR7 and TLR9 ligands [175]. Single nucleotide polymorphisms in the genes encoding TLRs have also been shown to confer a greater risk of developing infectious diseases. For example, the relatively common D299G polymorphism in the TLR4 receptor increases the risk of Gram negative infections [176], and is associated with an increased incidence of systemic inflammatory syndrome [177].

1.6.4 TLR expression and function in the intestine

In the intestine, although all TLRs are expressed at the mRNA level, the expression of only TLRs 1-5 and 9 has been confirmed at the protein level by a number of different visualisation techniques. TLR2 and TLR4 are known to be expressed at low levels in intestinal epithelial cells [178-180], while TLR3 expression is abundant in both the small and large intestine [181]. TLR5 expression is predominantly restricted to the colon [182, 183]. Intestinal epithelial cells are structurally and functionally polarised with an apical surface facing the intestinal lumen and a basolateral face facing the underlying basement membrane and there is evidence that TLRs are expressed in a spatially restricted fashion in the GI tract (Figure 1.6.4.1). For example, TLR2 and TLR4 were shown to be expressed on the basolateral surface of the ileal crypts [184] whilst TLR9 has been found to be apically expressed in the murine colon [185]. Furthermore, *in vitro*, polarised intestinal cancer cells respond differently depending on whether they are exposed to apical or basolateral CpGODN, demonstrating that TLR function, as well as expression, can be polarised [186]. Although studies using polarised human

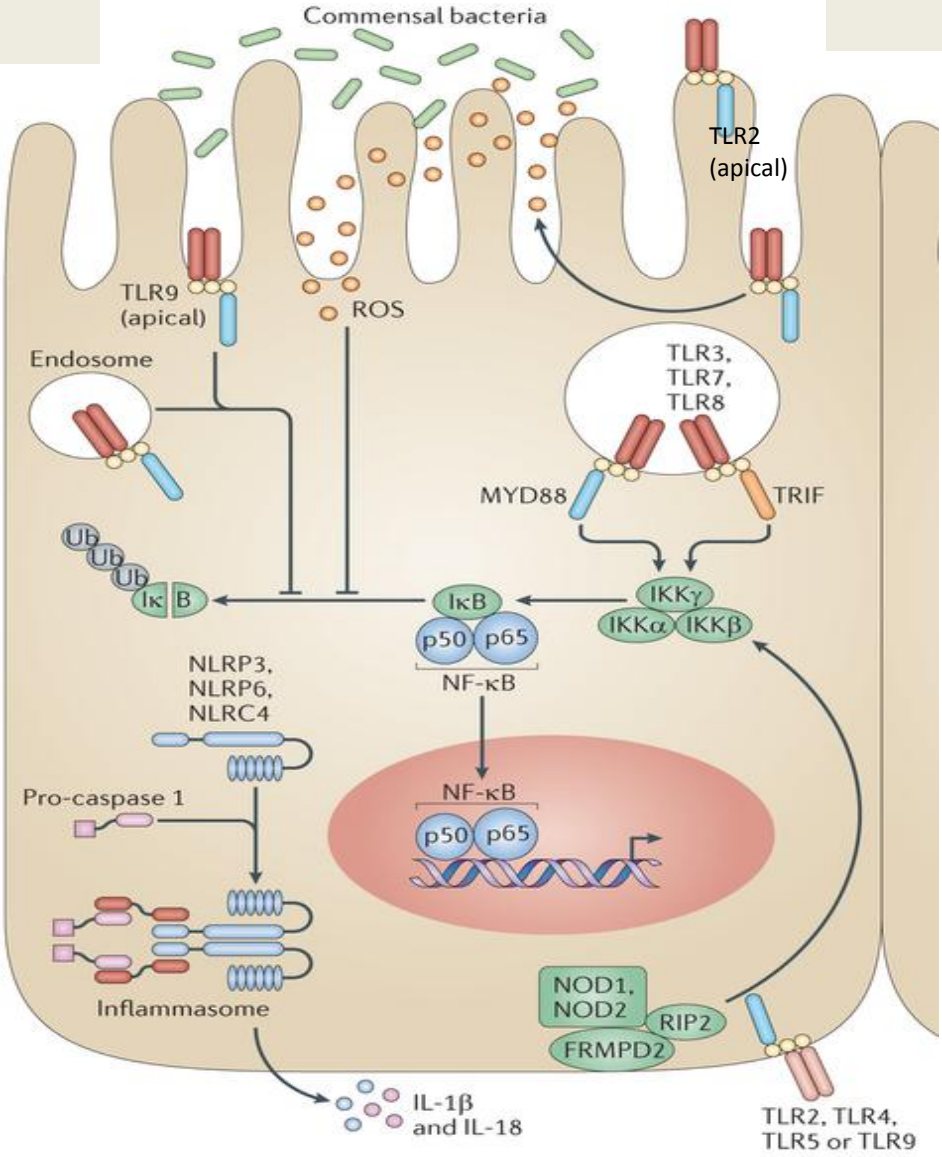


Figure 1.6.4.1 TLR expression in intestinal epithelial cells

TLRs are expressed in a spatially restricted fashion in IECs. The expression of TLR4, TLR5 has been shown on the basolateral surfaces of IECs whilst TLR2 and TLR9 are expressed on both the basolateral and apical surface of this cell type.

Polarized expression of TLRs at either the apical or basolateral membrane may contribute to the discrimination between commensal and pathogen microbial signals. For example, signalling through apical TLR9 promotes the inhibition of NF-κB signalling in IECs, whereas TLR signalling from the basolateral pole promotes NF-κB activation.

Adapted from 'Intestinal epithelial cells: regulators of barrier function and immune homeostasis.' Peterson and Artis, Nature Reviews Immunology 14, 141–153 (2014)

intestinal cancer cells indicated that TLR5 is expressed only on the basolateral surface of the intestine, recent studies have indicated that functional TLR5 is also present on the apical surface of the murine ileum and is capable of inducing the production of CXCL-1, an IL-8 homolog, in response to commensal derived Flagellin [183, 187].

The recognition of microbial products by TLRs leads to the induction of a variety of signalling pathways that regulate the host inflammatory response that is initiated upon pathogenic invasion. For example, studies have shown that intestinal cancer cells stimulation with Flagellin leads to the TLR5-mediated production of IL-8 and macrophage inflammatory protein-3 alpha (MIP3α), proinflammatory cytokines that are important in driving both the innate and adaptive immune responses to pathogenic organisms in the colon [188, 189]. However, given the constant presence of the commensal flora, intestinal cancer cells must be maintained in a state of immune tolerance. The expression of negative regulators of PRR signalling such as single Ig IL-1R-related molecule (SIGIRR) (also known as Tir8), Toll interacting protein (TOLLIP), peroxisome proliferator-activated receptor-γ (PPARγ) and interleukin-1 receptor-associated kinase monocytes/macrophages (IRAKM) by intestinal cancer cells, is critical in controlling the homeostasis and innate immune responses of the colon to enteric microflora. The mechanism of TLR inhibition varies between these proteins. For instance, SIGGIR, as an orphan receptor, binds to and therefore inhibits, TLR signalling by virtue of its TIR-domain [130]. By contrast, the mechanism by which TOLLIP down regulates TLR signalling is thought to be mediated by ubiquitination of the TLR4–CD14 complex [131]. Commensal signalling through TLRs has been shown to result in the upregulation of TLR inhibitory proteins such as PPARγ [190] and recent evidence has demonstrated a role for the hypo responsiveness afforded by TLR inhibition in host defence. The exaggerated inflammatory, antimicrobial and proliferative responses driven by the IL-1 receptor reported in SIGIRR deficient mice rapidly deplete the intestinal microbial community. This reduction in commensal gut microflora leads to a reduced ability to outcompete invading pathogens for space and nutrients, making the mice extremely susceptible to infection by *C. rodentium* and other intestinal bacterial pathogens. Thus, by sacrificing maximal innate responsiveness by

intestinal cancer cells to commensal bacteria, resistance against bacterial pathogens is promoted in the intestine [191].

In the GI tract, TLR signalling also regulates barrier function. For example, TLR2 activation has been shown to result in the reorganisation of Z01, a protein required for tight junction formation [192]. Treatment of intestinal cancer cells with TLR ligands *in vitro* resulted in increased transepithelial resistance, a measure of the strength of the tight junctions between intestinal cancer cells, suggestive of an increase in barrier function. Furthermore, recent data have demonstrated that sensing of commensal bacteria by TLRs is important for the actual spatial segregation of the microbiota [193, 194] with studies showing that TLR signalling from Paneth cells is vital for the secretion of antimicrobial peptides and lectins that prevent the invasion of pathogenic bacteria. Therefore, host intestinal cancer cells respond directly to the commensal microbiota through TLRs and enhance barrier function in a number of ways. In support of this, intestinal cancer cells-specific deletion of TLR4 and NOD1 or MyD88 impairs immunity to bacterial infections [195, 196]. Furthermore, although the majority of microbially-regulated genes involved in immune responses and barrier function are not dependent on the expression of MyD88 [197], MyD88 deficient mice demonstrate impaired barrier function [198] indicating the importance of MyD88 dependant signalling in the colon. MyD88 deficiency in some mouse strains also significantly alters the composition of the microbiota in a way that predisposes the conventionally raised host to viral infections. It has been speculated that MyD88-dependant increases in the expression of the antimicrobial genes, Reg β and Reg γ, contribute to the clustering of specific bacterial communities which are responsible for protection against viral infections [197]. In addition, TLR5 knockout (KO) mice develop spontaneous colitis and also metabolic abnormalities on some murine genetic backgrounds [199, 200]. Microbiota transfer from diseased TLR5KO mice into GF wild type mice reproduced the observed metabolic disease phenotype strongly suggesting that the microbiota are responsible for the increased insulin resistance and adiposity observed upon TLR5 deficiency [200]. This phenomenon is not restricted to TLR5, as TLR2KO mice have been reported to suffer a similar phenotype [201].

Ligation of TLRs on intestinal epithelial cells thus promotes the expression of antimicrobial peptides and regulates barrier function. Furthermore, TLR signalling

on intestinal cancer cells is vital for the development of the gastrointestinal immune system. In addition, TLR signalling can directly influence the commensal community which may have important consequences both in the prevention of pathogenic infection and also in the induction of disease in the colon and elsewhere.

1.6.5 TLR signalling in the intestine in disease

Given that TLRs induce the secretion of proinflammatory cytokines, dysregulation of TLR mediated signalling can lead to acute and chronic intestinal inflammation in the bowel, symptoms of which are characteristic of inflammatory bowel disease (IBD). Under healthy conditions, expression of the TLR 4 receptor complex is generally low in the intestinal mucosa [202-204], but is significantly upregulated in either nonactive and/or active human IBD colitis [204-206]. Additionally, the T-cell-derived cytokines, such as interferon gamma (IFNγ) and tumour necrosis factor alpha (TNF-α), which have been shown to play significant pathophysiological roles in IBD, upregulate intestinal epithelial TLR4 expression *in vitro* [203, 207], suggesting that changes in the commensal composition in A genetically susceptible host may therefore result in aberrant TLR4 hyper responsiveness of the intestinal mucosa [199].

Patients with IBD are at increased risk of developing colon cancer and the risk is proportional to the degree of inflammation [208]. Consistent with a role for TLRs in the development of colitis-associated cancer (CAC), TLR4KO mice are protected against tumour development in the colon induced by the carcinogen azoxymethane (AOM) followed by dextran sodium sulphate (DSS) administration [209], a commonly used mouse model of CAC. Furthermore, it was shown that TLR4 expression by intestinal cancer cells, rather than haematopoietic cells, was required for malignant transformation [210]. In support of this, increased TLR signalling by intestinal cancer cells was shown to increase the risk of inflammation-associated neoplasia [211, 212]. Moreover, SIGIRR KO mice develop increased inflammatory responses to AOM and DSS, and are more susceptible to CAC [211, 212]. As this phenotype can be rescued by the transgenic intestinal cancer cells expression of SIGIRR, the cancer phenotype of these mice was thought to result from dysregulated

TLR signalling in epithelial cells [211]. Furthermore, implanted TLR5-expressing colonic cancer cells stimulated by Flagellin, induced anti-tumour immunity and decreased tumour size, whereas knockdown of TLR5 expression resulted in increased tumour size [213]. Together, these studies suggest that intestinal cancer cells-mediated TLR signalling leads to increased inflammation, potentially promoting the development of inflammation-associated neoplasia in the intestine.

The effect of decreased PRR signalling on tumour number and size in the Min mouse model has been studied by crossing $Apc^{Min/+}$ mice with MyD88-deficient ($MyD88^{-/-}$) mice. Although the overall incidence of intestinal tumours was similar in $Apc^{Min/+}$ mice and $MyD88^{-/-}/Apc^{Min/+}$ mice, the tumours were significantly smaller in the absence of MyD88 [214], suggesting that intestinal PRR signalling may contribute to the development of sporadic colon cancer [215].

There are also many reports suggesting that tumours are supported by the host through the recruitment of inflammatory cells that are generally immunosuppressive, including regulatory T cells (Tregs) and myeloid-derived suppressor cells (MDSCs), which can dampen the otherwise the proinflammatory immune response [216]. In support of this, TLR4 activation on colonic tumour cells has been shown to have a protective effect against anti-tumour cytotoxic T cells and NK cells resulting in increased tumour burden [217].

1.7 Crosstalk between the Fas and TLR signalling pathways

Recent studies have provided evidence of a link between the Fas and TLR signalling pathways. Although Fas-mediated activation has been reported to directly activate key transcription factors and signalling pathways in order to induce the secretion of pro-inflammatory cytokines [218-221], several lines of evidence suggest that MyD88 may play a role in Fas-mediated inflammation. For example, it was found that in macrophages, Fas-induced chemokine release was abrogated in the absence of MyD88. Furthermore, *in vivo*, disrupting MyD88 signalling led to attenuated neutrophil in response to the murine agonistic Fas antibody, Jo-2 [222]. In addition, MyD88 silencing *in vitro* has also been shown to result in the attenuation of chemokine (C-X-C motif) ligand 1(CXCL-1) release from alveolar epithelial cells in

response to Jo-2 *in vivo* confirmatory studies demonstrating that MyD88-null mice are protected from Fas-induced acute lung injury [108].

In support of Fas playing a role in TLR-mediated inflammation, peritoneal macrophages from *lpr* or *gld* mice have been shown to have a diminished ability to produce IL-6 in response to LPS [104]. These *lpr* mice exhibit suppressed LPS- and IL-1- induced NF-κB activation and cytokine expression suggesting that Fas ligation enhances IL-R1/TLR4 signalling to promote macrophage-mediated inflammation. Moreover, interruption of Fas ligation was shown to suppress IL-1R1 and TLR4-induced IκBlα degradation in primary macrophages [104], suggesting that Fas ligation is able to modulate macrophage cytokine production by activation of NFκB.

Further evidence for a role of Fas in TLR-mediated inflammation came from studies that suggested that FADD can interact with MyD88 and in doing so, suppress TLR-mediated cytokine production. In the absence of Fas signalling, FADD was shown to be present in the cytoplasm of macrophages bound to MyD88, potentially blocking/limiting MyD88 signalling [104, 223]. Following activation of Fas, FADD is recruited to the DISC which may sequester FADD away from MyD88, thereby promoting TLR-mediated inflammation (Figure 1.3.3.1). Despite this compelling evidence, to the best of my knowledge, no study has directly investigated the crosstalk between the Fas and TLR signalling pathways in intestinal cancer cells.

1.8 Aims

Therefore the aims of this study were to:

1. Investigate the role of Fas signalling in intestinal inflammation and in particular the crosstalk between Fas and the TLR signalling pathways in intestinal cancer cells (Chapters 3 and 4).

2. Given that chronic inflammation in important in cancer and that ligation of Fas can lead to the production of inflammatory cytokines and chemokines, I sought to investigate the role of Fas in colon cancer (Chapter 5).

3. Furthermore, given the studies showing potential crosstalk between Fas and TLR4 in macrophages, and the studies showing that TLR4 antagonism is beneficial in colon cancer, I sought to assess whether a reduction in tumour-expressed Fas has the potential to increase the efficacy of TLR4 antagonism (Chapter 5).

2. Material and Methods

2.1 Materials

All reagents were stored and prepared according to the manufacturer's guidelines.

2.1.1 Bacterial strains

Escherichia coli strain K12 was obtained from the Alimentary Pharmabiotic Centre (University College Cork, Ireland). *Listeria monocytogenes* strain EGD (serotype 1/2a) was a gift from Professor C Hill (University College Cork). *Salmonella typhimurium* strain SJW1103 (wild type) was a gift from Professor P. O'Toole.

2.1.2 Cell lines and tissue

HT29, HCT116, CT26, SW480, Jurkat T, HL-60 and THP-1 cells were obtained from the American Type Culture Collection (MD, USA). Tissue from C57BL/6 TLR2, 4 and 5 knockout (KO) and WT mice was kindly provided by the Alimentary Pharmabiotic Centre (University College Cork) whilst tissue from C57BL/6 MyD88 and TRIF KO mice was obtained from P. Fallon Trinity College (Dublin, Ireland). These mice were originally generated by the Yale School of Medicine (TLR5 KO) or the Shizuo Akira laboratories (TLR2, TLR4, MyD88 and TRIF KO). Briefly, the *tlr2* [224], *tlr4* [225], *tlr5* [226], *myd88* [161], and *trif* [170] genes were disrupted by homologous recombination replacing part or whole of the open reading frame of the relevant gene in E14.1 embryonic stem (ES) cells. ES cell clones were selected in the presence of neomycin and ganciclovir. ES cell lines containing a mutant allele were microinjected into C57BL/6 blastocysts and heterozygous mice were intercrossed to produce TLR2, 4, 5 or TRIF or MyD88-deficient mice. Mutant mice were born at the expected Mendelian ratio, were heatlhy and did not show any obvious clinical or behavioural abnormalities. Northern blot analysis was performed to confirm that the mutations inserted inactivated the relevant genes.

Tissue from Swiss Webster wild-type (WT) and germ-free (GF) mice was kindly provided by the Alimentary Pharmabiotic Centre (University College Cork). GF mice were generated, colonized for 49 days, and tissue obtained.

2.1.3 Mice

Six week old female Balb/C mice were obtained from Harlan (Oxon, UK) and maintained in the animal facility of University College Cork. Standard housing and environmental conditions were maintained (temperature 21°C, 12 hrs light and 12 hrs darkness with 50% humidity). Animals were fed sterile standard pellet diet and water *ad libitum*. Animal husbandry and experimental procedures were approved by the University College Cork Animal Experimentation Ethics Committee (AEEC).

2.1.4 Reagents

2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one hydrochloride (LY294002) *L9908* (Sigma Aldrich, Dublin, Ireland)

4-[5-(4-Fluorophenyl)-2-[4-(methylsµlfonyl)phenyl]-1*H*-imidazol-4-yl]pyridine (SB203580) *S8307* (Sigma Aldrich)

2-[(Aminocarbonyl)amino]-5-(4-fluorophenyl)-3-thiophenecarboxamide (TPCA-1) *T1452* (Sigma Aldrich)

Agarose *A9414* (Sigma Aldrich)

AgeI *R0552S* (New England Biosciences, MA, USA)

Bovine Serum Albumin *A9418* (Sigma Aldrich)

BCA Protein Assay Reagents A and B *23223* and *23224* (Thermo Scientific, IL, USA)

Brain-heart infusion broth *53286* (Sigma Aldrich)

Dulbecco's Modified Eagle Medium *D5796* (Sigma Aldrich)

CCL-2 *571402* (Biolegend, CA, USA)

Collagenase Dispase solution *10269638001* (Roche Diagnostics, Basel, Switzerland)

Crystal Violet Dye *C3886* (Sigma Aldrich)

Dimethyl sulphoxide *D2650* (Sigma Aldrich)

DPX mounting medium *44581* (Sigma Aldrich)

EcoRI *R0101S* (New England Biosciences)

Eosin *45260* (Sigma Aldrich)

Ethyl (6R)-6-[N-(2-Chloro-4-fluorophenyl)sulfamoyl]cyclohex-1-ene-1-carboxylate (TAK242) *tlrl-cli95* (Invivogen, Toulouse, France)

Ultra-pure FL-AST *tlrl-epstfla* (Flagellin) (Invivogen)

Donkey serum *D9663* (Sigma Aldrich)

Gentamycin sulphate salt *G1264* (Sigma Aldrich)

Normal Goat serum *X0907* (DAKO Diagnostics)

Human recombinant IL-8 *14-8089-63* (eBioscience, CA, USA)

Heat-inactivated foetal calf serum *F2442* (Sigma Aldrich)

Haematoxylin *51260* (Sigma Aldrich)

Immobilon Western Chemiluminiscent HRP substrate *WBLUF0500* (Merck Millipore, MA, USA)

Ultra-pure LPS *tlrl-pb5lps* (Invivogen)

Anti-fade fluorescent mounting media *GM-304* (Dako)

Luria-Bertani broth *L3152* (Sigma Aldrich)

Pam3CSK4 *tlrl-pms* (Invivogen)

Penicillin/Streptomycin *P4333* (Sigma Aldrich)

Peptidoglycan – *S. aureus tlrl-pgnsa* (Invivogen)

Phorbol 12-myristate 13-acetate (PMA) *P1585* (Sigma Aldrich)

Phosphate Buffered Saline *D8662* (Sigma Aldrich)

Polyinosinic-polycytidylic acid sodium salt *tlrl-pic* (Invivogen)

Polyoxyethylene(20)sorbitan monolaurate (TWEEN) *P1379* (Sigma Aldrich)

Polybrene *sc-134220* (Santa Cruz, Heidelberg, Germany)

Protease inhibitor cocktail I *539131* (Merck Millipore)

Puromycin *P8833* (Sigma Aldrich)

Oligodeoxynucleotide 2006 *tlrl-2006* (Invivogen)

Oligodeoxynucleotide 1826 *tlrl-1826* (Invivogen)

Tet-pLKO-puro plasmid *#21915* (Addgene, MA, USA)

Red cell lysis buffer *R7757* (Sigma Aldrich)

Resazurin powder *R7017* (Sigma Aldrich)

Tumour necrosis factor alpha *300-01A* (PeproTech, NJ, USA)

Triton X *T8787* (Sigma Aldrich)

Staurosporine from *Streptomyces* sp. *S5921* (Sigma Aldrich)

Xho1 *R0146S* (New England Biosciences)

Table 2 Antibodies used

Target	Dilution/ Conc.	Species raised against	Clone	Host	Source
Fas	50μg/ml	Human	CH-11	Mouse	Millipore
Fas	100μg/ml	Murine	Jo-2	Rabbit	BD Biosciences
Fas	1 in 1000	Human	C-20	Rabbit	Santa Cruz
Fas	1 in 500	Murine	X-20	Rabbit	Santa Cruz
FasL	1 in 1000	Murine	Ab15285	Rabbit	Abcam, Cambridge UK
TLR4	1 in 500	Human	H-80	Rabbit	Santa Cruz
TLR5	1 in 500	Human	H-127	Rabbit	Santa Cruz
TLR2	1 in 1000	Human	N/A	Rabbit	Novus
TLR9	1 in 1000	Human	N/A	Rabbit	Novus
IGFR-β1	1 in 1000	Human	C-20	Rabbit	Santa Cruz
TNFR-1	1 in 1000	Human	C-20	Rabbit	Santa Cruz
β actin	1 in 5000	Human	N/A	Rabbit	Sigma Aldrich
FADD	1 in 200	Human	H-181	Rabbit	Sigma Aldrich
MyD88	1 in 200	Human	N/A	Rabbit	Santa Cruz
IRAKm	1 in 500	Human	N/A	Rabbit	Abbomax, CA USA
SIGIRR	1 in 500	Human	N/A	Rabbit	Pro-Sci, CA USA
Lactoferrin	1 in 200	Murine	N-20	Goat	Santa Cruz
F4/80	1 in 1000	Murine	N/A	Rabbit	Santa Cruz
FLAG	1 in 200	Human	F1804	Rabbit	Sigma Aldrich

Table 3 Oligonucleotide Primers and Universal probe Library probes

Gene Name	Forward	Reverse	UPL probe #
TOLLIP	caacctcgtcatgtcctacg	gctggtacactgttggcatc	38
SIGIRR	tgaaagacgggcttccatt	gacaggttggccttgacc	75
PPARγ	gacaggaaagacaacagac aaatc	ggggtgatgtgtttgaacttg	7
IRAKm	agagctctgcgctgttctg	gctgcttgaaagtctctctgc	26
CXCL-1	tcctgcatcccccatagtta	tcctgcatcccccatagtta	52

Table 4 Applied Biosystems® TaqMan® Probes

Gene Name	Probe
Fas	Hs00236330_m1
FasL	Mm01292781_m1
TNFα	Hs00174128_m1
IL-8	Hs00174103_ml

2.2 Methods

2.2.1 Cell culture

Cells were cultured in 75cm^2 flasks at 37°C in 5 % $C0_2$ in DMEM supplemented with 10% FBS and 10,000 units/ml penicillin, 10mg/ml streptomycin. Cells were routinely grown to 70-80% confluence in T150 cell culture flasks before bi-weekly passaging. Passaging involved washing cells with pre-warmed PBS and subsequent incubation with 3 ml EDTA to detach cells from the flask wall.

2.2.2 Western blotting

Cells were seeded at 1x10^5 cells/ml into 6 well plates and then cultured until 60% confluent. Cells were then treated as specified in the figure legends. Following treatment cells were washed with ice-cold PBS and then lysed on ice for 1 hr with 100ml of lysis buffer containing 50 mM Tris-HCl (pH 8.0), supplemented with 1X protease inhibitor cocktail (Merck Millipore, USA). Cells were then scraped and transferred to a 1.5ml eppendorf. Lysates were centrifuged at 14600 rpm at 4°C for 10 mins. The resulting cell debris was discarded and lysate was stored at -20°C.

Protein standards were prepared using Bovine Serum Albumin (BSA) (Thermo Scientific, IL, USA) (0, 2.5, 5, 7.5, 10, 15, 20µg/ml) and added to a 96 well plate. 2µl of each sample was added to the plate followed by 38µl of dH_20. Both standards and samples were analysed in triplicate. 160µl of BCA Protein Assay Reagent (Thermo -Scientific) was added to each well, the plate agitated and then left at 37°C for 30 mins before reading at 595nm on a spectrophotometer.

The appropriate amount of cell supernatant containing 50µg of protein was mixed with 5 x PAGE loading buffer (125mM Tris, 2% SDS, 20% Glycerol, 2.5% beta mercaptoethanol) and lysis buffer (containing 50 mM Tris-HCl (pH 8.0) to a final volume of 20µl. Samples were boiled for 5 mins before loading onto a separating and stacking SDS gel. A 19-180 kilo Dalton (kDa) molecular weight marker (Sigma Aldrich) was run alongside the samples. Proteins were separated by electrophoresis at 40mA and then transferred overnight onto an Immobilon –P polyvinylidene diflouride membrane at 40V and 4°C using a wet transfer method. The following

morning, membranes were stained with Ponceau to check for transfer of proteins. Ponceau was washed with distilled water and PBS-0.1% TWEEN before membranes were blocked using 5 % (w/v) milk powder in PBS-0.1% TWEEN (hereafter referred to as blocking buffer) for 1 hr with rocking. Membranes were washed for 5 mins in PBS-0.1% TWEEN before the appropriate primary antibody was added diluted in blocking buffer and stored overnight at 4°C. Membranes were washed 3 times with PBS-0.1% TWEEN for 10 mins at a time, and then incubated for 1 hr at room temperature with the appropriate secondary antibody. Finally, membranes were washed 3 times with PBS-0.1% TWEEN for 5 mins at a time before detection using an Immobilon Western Chemiluminiscent HRP substrate according to manufacturer's instructions. Protein bands were analysed using ImageJ (National Institutes of Health, Bethesda, MA, USA, http://imagej.nih.gov/ij/, 1997-2012.). Changes in protein expression were determined after normalising the band intensity of each lane to that of β-actin.

2.2.3 RNA extraction

Cells were lysed with 250µl of RNA lysis buffer and RNA extracted using the Bioline ISOLATE kit according to the manufacturer's instructions. Resultant RNA was quantified using a Nano drop.

2.2.4 cDNA preparation and quantitative real-time polymerase chain reaction (qRTPCR)

10 µl of total RNA was used as the template for cDNA synthesis using a commercially available cDNA synthesis kit according to the manufacturer's instructions (Bioline, UK). This was added to 1µl of Oligo (Dt), and 1µl of 10Mm dNTP and heated to 65°C for 10 mins. Following 2 mins on ice, 4µl of 5x Reverse Transcriptase Buffer, 1µl of RNase inhibitor, 0.25µl of Reverse Transcriptase, and 2.75µl of DEPC treated water was added to each sample before incubation at 37°C for 30 mins. The reaction was terminated by a final incubation at 70°C for 15 mins

and chilling samples on ice. 2µl of cDNA template was amplified in a 25µl total reaction volume as per Applied Biosystems standard PCR protocol using appropriate probes and TaqMan Gene Expression Master Mix (Applied Biosystems, UK). Thermal cycling was performed in a machine (Applied Biosystems), with general conditions as follows: 50 °C for 2 mins; 95 °C for 10 mins; 60 cycles at 95 °C and 60 °C for 1 min. The results were analysed using the ΔΔ ct method and the gene of interest was normalized to the corresponding GAPDH results. Data were expressed as fold induction relative to untreated.

2.2.5 Resazurin Assay

Resazurin powder (Sigma Aldrich) was hydrated with PBS under sterile conditions to make a 10 x stock solution (440µM). The solution was filter sterilized using a 0.22µm filter and stored in a foiled covered container at 4°C until use. Media was aspirated off the 6 well plates and each well was washed once with PBS. 2ml of pre-warmed 1x Resazurin solution was added to each well before measuring fluorescence at 535-590nm on a GenIOS fluorometer. Fluorescence readings were taken at a variety of time points (5-55 mins) to create a standard curve of fluorescence against time. Readings were subsequently taken during the log phase of the reaction.

2.2.6 Caspase 3/7 Assay

Cells were seeded overnight in black flat-bottomed 96-well plates at a density of 20,000 cells/well, treated with CH-11 for 1hr and subsequently with 100ng/ml Flagellin or 100ng/ml LPS for 24 hr, or were treated with each agonist separately. Apo-ONE caspase-3/7 reagent was added and following 1 hr incubation, fluorescence (485 excitation, 530 emission) was measured using a GENios Microplate Reader (Tecan Group Ltd, Männedorf, Switzerland). Jurkat T cells were treated with 200ng/ml CH-11 as a positive control. Changes in caspase 3/7 activation were normalised relative to untreated cells.

2.2.7 Immunohistochemistry

Formalin fixed and paraffin embedded murine colonic sections were deparaffinized and rehydrated using Xylene and an ethanol gradient (100%, 95%, 70% ethanol and then water). The sections were then boiled with sodium citrate (pH 8) in order to induce epitope retrieval. Sections were washed in Tris Saline solution (1% Tris Saline (0.05M) and 0.001% Saponin) hereafter known as wash solution, and blocked in 3% hydrogen peroxide. Following a further wash in 1% normal goat serum (NGS) or normal rabbit serum (NRS) Wash solution, a blocking step in 5% NXS Tris Saline and a final wash, sections were incubated with primary antibody in 1% NXS Tris Buffer over night at 4°C. The following morning, sections were washed with wash solution before incubation for 45 mins at room temperature with 0.5% biotinylated anti-Rabbit IgG or anti-goat IgG (Vector Laboratories, Inc CA, USA). A five minute wash step followed, after which the sections were incubated with ABC Elite complex (Vector Laboratories) for 45 mins at room temperature. Sections were then washed and DAB substrate added (Vector Laboratories) according to manufacturer's instructions. Finally sections were washed in tap water, counterstained with Mayer's haematoxylin (Sigma Aldrich), washed in tap water again before being mounted with crystal mount (Sigma Aldrich). The specificity of the reaction was proven by staining with a normal rabbit IgG or normal goat IgG isotype control.

2.2.7 Enzyme Linked Immunoassay (ELISA)

IL-8 levels were quantified using the Human IL-8 ELISA Ready-SET-Go!® kit (eBiosciences, San Diego, USA) according to manufacturer's instructions.

2.2.8 Immunofluorescence

Cells were seeded at $1x10^5$cell/ml onto EZ slides (Millipore) before fixing in methanol the following day. Cells were permeabilised using 0.2% Triton X-100

before blocking in 10% normal donkey serum. The slide was incubated with primary antibody overnight in 1.5% normal donkey serum at 4°C. The following day, the slide was incubated in the appropriate secondary antibody diluted in normal donkey serum for 1 hr before counterstaining with DAPI and mounting with anti-fade fluorescent mounting media. Specificity of the reaction was proven by incubating with only primary or only secondary antibody.

2.2.9 Cloning

The pLKO-Tet-On vector was transformed into competent *E.coli* cells and streaked onto an ampicillin selective (50μg/ml) plate. Following 24 hr incubation at 37°C, colonies were expanded, maxi-prepped and DNA purified using the Qiagen Maxi Plus Plasmid kit. The purified plasmid was digested with *AgeI* and *EcoRI* for 1 hr The digested plasmid was run on a 2% agarose gel to confirm excision of the 'stuffer' fragment and subsequently gel purified using the QIAquick Gel Extraction Kit. Oligomer sequences for shRNA against Fas were reconstituted and used in the subsequent ligation reaction with the pLKO-Tet-On purified plasmid at a final concentration of 0.1nml/μl. *Stb13* cells were transformed with 4μl of ligation product, incubated on ice for 30 mins followed by heat shock for 45 seconds at 42°C. Cells were put on ice for 2 mins and 250μl of super optimal broth (SOC) media was added to each vial. The SOC innoculum was left shaking at 225rpm at 37°C for 1 hr and 100μl of was spread onto prewarmed agar plates containing 50μg/ml carbenicillin. *Stb13* cells were also transformed with the pUC19 control plasmid to confirm transformation efficiency. After 48 hrs incubation at 37°C, colonies were grown in LB broth overnight containing 50μg/ml ampicillin and the following day mini-prepped using the Qiagen Miniprep kit. The purified plasmids were digested with *XhoI* for 1 hr before running the digestion products on a 2% agarose gel.

2.2.10 Viability

100µl of cell suspension was added to 900µl of Trypan Blue and 20µl was transferred to a haemocytometer. The number of stained cells was counted, in addition to the total number of cells and percentage viability assessed.

2.2.11 Coimmunoprecipitation

Initial optimisation experiments were performed by transfecting HCT116 cells with either 0.5, 1 or 2µg HA-tagged MyD88 constructs or with 1, 2 or 5 µg of FLAG-tagged FADD constructs and protein expression detected by Western blotting. The coimmunoprecipitation reaction was subsequently optimised by co-transfecting HCT116 cells with 2µg HA-tagged MyD88 and 5 µg FLAG-tagged FADD constructs. 25µl of anti-HA or anti FLAG was added to 100µl protein A agarose beads and incubated overnight at $4^{o}C$ with agitation. Protein A agarose beads complexed with the relevant antibody was added to lysate and incubated at room temperature for 2 hrs. The agarose bead/Ab complex was washed x2 with TBS and x2 with RIPA buffer. Lysate-agarose bead suspension was then washed x1 in 500µl water, with 100µl Laemmli buffer added. Samples were then heated to $95^{o}C$ for 5mins and run on a 15% SDS PAGE gel, transferred to PVDF membrane and immunoblotted with the relevant antibody.

2.2.12 dHL-60 migration assay

HL-60 cells were cultured in $75cm^2$ flasks with 1.3% DMSO for 5 days. dHL-60 cells were then seeded at $1.5x10^6$ cells/ml in the upper chamber of a 5µm pore size transwell. 750µl of supernatant derived from either untreated $HT29^{scr\ shRNA}$ or $HT29^{Fas\ shRNA}$ cells, or from cells treated for 24 hrs with CH-11, Flagellin or LPS, was added to the lower chamber.

In order to control for dHL-60 cells migrating to LPS, Flagellin or CH-11 in the media, media alone or media conditioned with CH-11, Flagellin, LPS was added to the lower chamber. 20nM hrIL-8 was added in media to the lower chamber as a positive control. After 2 hrs cells that had migrated through the insert were immobilized onto a glass slide, fixed and stained by H&E. Cells from 10 fields of view (x40) from each slide were counted and averaged.

2.2.13 THP-1 derived macrophage migration assay

THP-1 cells were seeded at $2x10^5$ cells/ml into the upper chamber of a 5µm pore size transwell in 10% FCS RPMI containing 10µM PMA. 750µl of 10% FCS RPMI was added to the bottom chamber of the transwell. 72 hrs later, the media in the bottom chamber was replaced with supernatants from SW480 cells, either untreated, or stimulated with CH-11, Flagellin, or LPS. In order to control for THP-1 derived macrophages migrating to LPS, Flagellin or CH-11 in the media, media alone or media conditioned with CH-11, Flagellin, LPS was added to the lower chamber. 54nM CCL2 was added in media to the lower chamber as a positive control. After 6 hrs incubation at 37^oC, the insert was cut out of the upper chamber, washed twice in PBS and fixed in ice cold methanol for 5 mins. Inserts were then stained in 0.1% crystal violet in 0.1M borate pH 9.0 and 2% ethanol for 20 mins. Inserts were then washed 3 x 5 mins in PBS and placed into a 24 well plate containing 200µl of acetic acid to elute the stain. Absorbance was read at 570nm.

2.2.14 Generation of Fas $^{Low/negative}$ intestinal cancer cells

Cells were transfected with lentiviral particles containing target-specific shRNA against Fas (sc-29311-V (human) or sc-29312-V (murine)) or control lentiviral particles containing scrambled shRNA (sc-108080) (Santa Cruz Biotechnology), according to the manufacturers' instructions. Cells were seeded in 12-well plates at a concentration of $7.5x10^3$ cells/ml. Cells were infected 24hrs later with lentiviral particles in the presence of 4µg/ml polybrene, and cultured in selection medium containing 6-8µg puromycin until resistant clones could be identified. Resistant

clones were selected by limiting dilution. Knockdown of Fas expression was determined by Western blotting and functional analysis.

2.2.15 Bacterial cell culture and infection

L. monocytogenes was grown to the logarithmic growth phase in brain–heart infusion broth at 37°C shaking at 200 rpm, while *S. typhimurium* were grown in Luria–Bertani broth. Bacteria were diluted in PBS for infection at multiplicity of infection (MOI) of 10:1. Intestinal cancer cells were seeded overnight at $5x10^5$cells/ml and cultured with *L. monocytogenes* or *S. typhimurium* for 3hr Supernatant was removed and replaced with gentamycin-containing media (50ng/ml), and 24 hrs later cell culture supernatant was harvested. SW480 cells were seeded overnight at $5x10^5$ cells/ml, stimulated for 1 hr with CH11(100ng/ml) and cultured with *E. coli* K-12 strain (10:1,bacteria:cells) for 8hrs. Cells were lysed for subsequent RT-PCR analysis.

2.3 *In vivo* studies

For the initial *in vivo* study, female Balb/c mice were injected with either $CT26^{scr\ shRNA\#1}$ (n=4) or $CT26^{Fas\ shRNA\#1}$ cells (n=4) and tumour growth monitored over a period of 21 days. A separate control group were injected with PBS only (n=3)

The subsequent investigation compared two $CT26^{FasshRNA}$ groups each with one control $CT26^{Scr\ shRNA}$ group. Data from the initial investigation indicated that the mean survival time (calculated as number of days to tumour growth) of the control group is 21 days (day at which the experiment was terminated) whilst mice injected with $CT26^{FasshRNA}$ cells developed tumours after 9 days. In order to reject the null hypothesis that the survival curves from the $CT26^{FasshRNA}$ and $CT26^{Scr\ shRNA}$ –derived tumours are the same with probability 0.800, 6 mice per $CT26^{FasshRNA}$ and $CT26^{Scr\ shRNA}$ group were required (n=6). The type I error associated with this test is 0.05. A separate control group were injected with PBS only (n=2).

2.3.1 Treatment groups and ear marking

Mice were weight matched and divided randomly into groups. Mice were ear clipped for identification purposes.

2.3.2 Monitoring body weight

Mice were weighed thrice weekly to monitor changes in body weight and for calculation of the appropriate dose of TAK242.

2.3.3 Tumour formation

Mice were subcutaneously injected into the right flank with CT26 cells suspended in 100µl of PBS. Tumour formation was monitored thrice weekly by palpation at the site of injection. The width (w) and length (l) of the tumours was measured using Vernier calipers and the mean tumour volume calculated using the formula:

$\frac{1}{2}$ (l x w^2).

2.3.4 Drug administration

TAK242 was administered at 1mg/kg bi-weekly in 100µl of sterile PBS to mice via intraperitoneal injection. Alternate sides of the abdomen were injected to prevent inflammation by repeated injection at one site and no adverse effects were observed upon injection. Assessments of the level of suffering of the animals under study was undertaken by way of a distress scoring sheet taking into account appearance, food and water intake, behaviour and any clinical indication of distress. No side effects were noted in those mice administered with TAK242.

2.3.5 Tumour sampling

Following euthanasia by CO_2 inhalation and cervical dislocation, tumours were excised from mice and dissected for subsequent analysis using a clockface dissection

method. This method ensured that all stored tissue incorporated tumour tissue from the core to the leading tumour edge. Tissue was stored in liquid nitrogen for Western blotting and formalin for immunohistochemistry. In addition, tissue was placed in an eppendorf containing 1 ml of a working solution of collagenase/dispase in PBS on ice.

2.3.6 Single cell suspension of tumour cells

Tumours in collagenase/dispase solution were incubated at 37^{o}C for 1hr with shaking. Tumour tissue was placed into a petri dish, 10ml of serum free DMEM added and cells were then passed through a cell strainer. Cells were pelleted, washed in PBS and resuspended in 2ml of Red Cell Lysis Buffer. Following incubation at room temperature for 10 mins, 10% FCS DMEM media was added to stop the lysis reaction. Finally, a cell count was performed to obtain a final concentration of 1.x 10^{6}cells/ml.

2.3.7 Flow cytometry

200,000 cells per tumour sample were re-suspended in 200µl of cell staining buffer and samples were incubated with monoclonal antibodies against CD4 (T helper), CD8 (Cytotoxic T cells), CD49b (Natural killer cells), SIGLEC-F (eosinophils), LY6G+ (neutrophils), and F4/80 (macrophages) for 30 minutes on ice in the dark. Cells were then washed twice with 2 ml of cell staining buffer and centrifuged at 350 x g for 5 minutes before being resuspended 0.5ml of cell staining buffer. 2.5ug/ml of ViViD viability staining solution was added per million cells and sample incubated on ice for 15 minutes in the dark.

Debris was excluded from flow cytometric analysis by excluding those events with low forward scatter (FSC) and high side scatter (SSC) properties. Singlets were defined by having a similar height (FSC-H) and area (FSC-A) measurement in the forward scatter. Live cells were identified (ViViD^{neg}) and subsequently cell populations were assessed using the fluorescently tagged markers with Accuri C6 Flow Cytometer System and CFlow commercial software.

2.4 Statistical analysis

Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using One-way Anova with Tukeys post-test, or by students paired *t* test. Values of $p < 0.001$ are indicated by three asterisks (***). Values of $p < 0.01$ are indicated by two asterisks (**). Values of $p < 0.05$ are indicated by one asterisk (*).

Chapter 3

TLR 4 and TLR 5 upregulate Fas and FasL expression in intestinal cancer cells *in vitro* and in *vivo*

3.1 Introduction

The gastrointestinal tract, along with other mucosal surfaces of the body, such as the airways, skin, and reproductive tract are continuously exposed to innocuous environmental antigens and commensal microorganisms that live in a mutually beneficial relationship with their mammalian hosts. In fact the adult human intestine is home to an estimated 10^{14} commensal bacteria [227] and the microbial colonisation of the colon has been found to essential for the development and function of the gut associated lymphoid tissue (GALT). For example, commensal bacteria have been shown to offer protection against tissue injury, as well as promoting angiogenesis [228-230] and essential for the maintenance of intestinal barrier function [231, 232]. In addition, commensals also aid in digestion, as well as in the absorption and storage of nutrients [6, 233, 234]. Mammalian intestinal epithelial cells (IECs) have therefore evolved to be broadly immunologically hyporesponsive to the commensal bacteria to which they are constantly exposed. They do this by a variety of mechanisms such as the limited and restricted localisation of expression of pathogen recognition receptors (PRR) (reviewed in [235]) as well as the upregulation of negative regulators of PRR signalling such as Single Ig IL-1-related receptor (SIGIRR) [236].

As crucial innate immune sensors in the intestine, studies of the family of PRRs known as the Toll-like Receptors (TLRs) have shown that the characteristic signal transduction pathways downstream of these receptors in intestinal cancer cells have both inflammatory and homeostatic roles following binding of their cognate ligands [237]. In the healthy colon, basal activation of TLRs, along with other PRRs, by the commensal bacteria, maintains barrier function and the crosstalk between intestinal cancer cells, intestinal microbes and local immune cells is a fundamental feature of intestinal homeostasis. Colonisation of the colon, however, carries the risk of infection and inflammation if epithelial or immune cell homeostasis is disrupted and so TLR signalling in the intestine is necessarily tightly controlled [235].

Fas (CD95/APO-1) is a member of the tumour necrosis factor (TNF) superfamily. Members of this family function in a plethora of different cellular responses, including cellular differentiation, proliferation, and migration. Fas is best known, however, for its role in apoptosis which is triggered upon binding of its cognate

ligand, FasL. Fas receptor engagement induces the formation of a death-inducing signalling complex, resulting in the cleavage and activation of pro-caspase-8. Caspase-8, in turn, activates a caspase cascade, culminating in the apoptotic death of the Fas-bearing cell. Fas is ubiquitously expressed throughout the human body, but is particularly abundant activated mature lymphocytes, in the liver, heart, brain, and colon tissues. Studies have shown that Fas is constitutively expressed within the cytoplasm and at the basolateral surface of every colonic epithelial cell [238] irrespective of its location and early studies suggested that the Fas/FasL system was involved in the regulation of normal apoptotic cell turnover in epithelial crypts [119]. More recently, however, using mice deficient for Fas specifically in intestinal cancer cells, Fas has been shown to have a potentially cytoprotective role in a DSS model of experimental colitis, a result which is more suggestive of a protective rather than pro-apoptotic role in the intestine [147]. Indeed Fas has been shown to activate non-apoptotic signalling pathways in intestinal cancer cells such as JNK activation [239] suggesting that the non-apoptotic outcomes of Fas signalling may predominate in the intestine.

Fas expression is often modulated in diseases and pathological conditions. For instance, Fas is upregulated in Parkinson's disease [240] and is downregulated in many cancers [241, 242]. Moreover, expression of its ligand, FasL, has been shown to be increased in many cancers including that of the colon [128, 243]. Thus, dysregulation of the Fas/FasL system may contribute to pathologies of the colon.

Given that IECs constitutively express Fas and FasL, expression levels of which are often altered in pathological circumstances, the mechanism by which expression of these proteins is regulated was investigated. Studies have shown that activation of TLRs on macrophages can lead to an upregulation Fas expression [244-246], suggesting that stimulation of TLRs on intestinal cancer cells may also affect Fas and FasL expression. To date, however, no studies have investigated the potential of TLR ligands to modulate Fas and FasL expression in the colonic epithelia.

AIM – The aim of this chapter was to examine the effect of TLR ligands on the expression of Fas and FasL in human intestinal cancer cells.

3.2 Results

3.2.1 SW480, HCT116 and HT29 human intestinal cancer cells express TLR2, 4, 5 and TLR9

To investigate if TLR ligands are capable of upregulating Fas and/or FasL expression in intestinal cancer cells, basal TLR expression was examined. SW480 human intestinal cancer cells have been previously shown to express TLRs 1-9 [247]. As shown in Figure 3.2.1.1 a and b, expression of TLR4 and TLR5 was confirmed by immunofluorescence and Western blotting. Expression of TLRs 4 and 5 was also detected in two other cell lines examined, HCT116 and HT29. Moreover, all three cell lines were shown to express TLRs 2 and 9 (Figure 3.2.1.1b).

3.2.2 Fas and FasL are expressed in SW480 and HT29 human intestinal cancer cells

The basal level of Fas and FasL expression in SW480 cells was investigated. As shown in Figure 3.2.2.1, SW480 cells express a basal level of both Fas and FasL throughout the cytoplasm. HT29 cells were also confirmed to express Fas and FasL (Figure 3.2.2.1b).

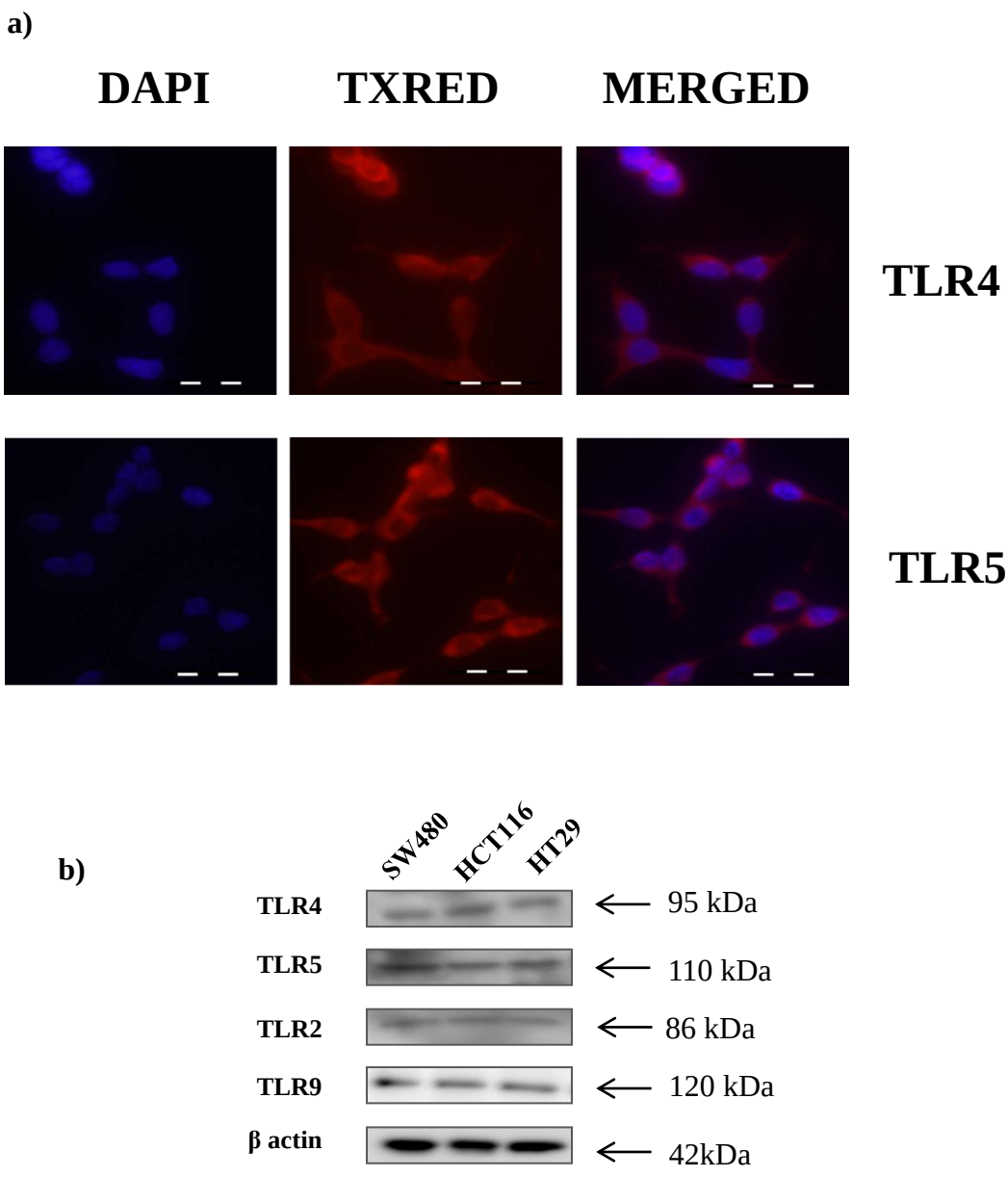


Figure 3.2.1.1 Colonic epithelial cells express TLR4 and TLR5. **(a)** SW480 cells were seeded at $1x10^5$cells/ml onto sterile coverslips. 24 hrs later, cells were fixed and immunofluorescently stained with anti-TLR4 or anti-TLR5 antibodies. Cells were counterstained with DAPI. Scale bar = 100μM. **(b)** Cell lysates were separated by SDS-PAGE and probed with anti-TLR 2,4,5 or 9 antibodies. β actin was used as a loading control.

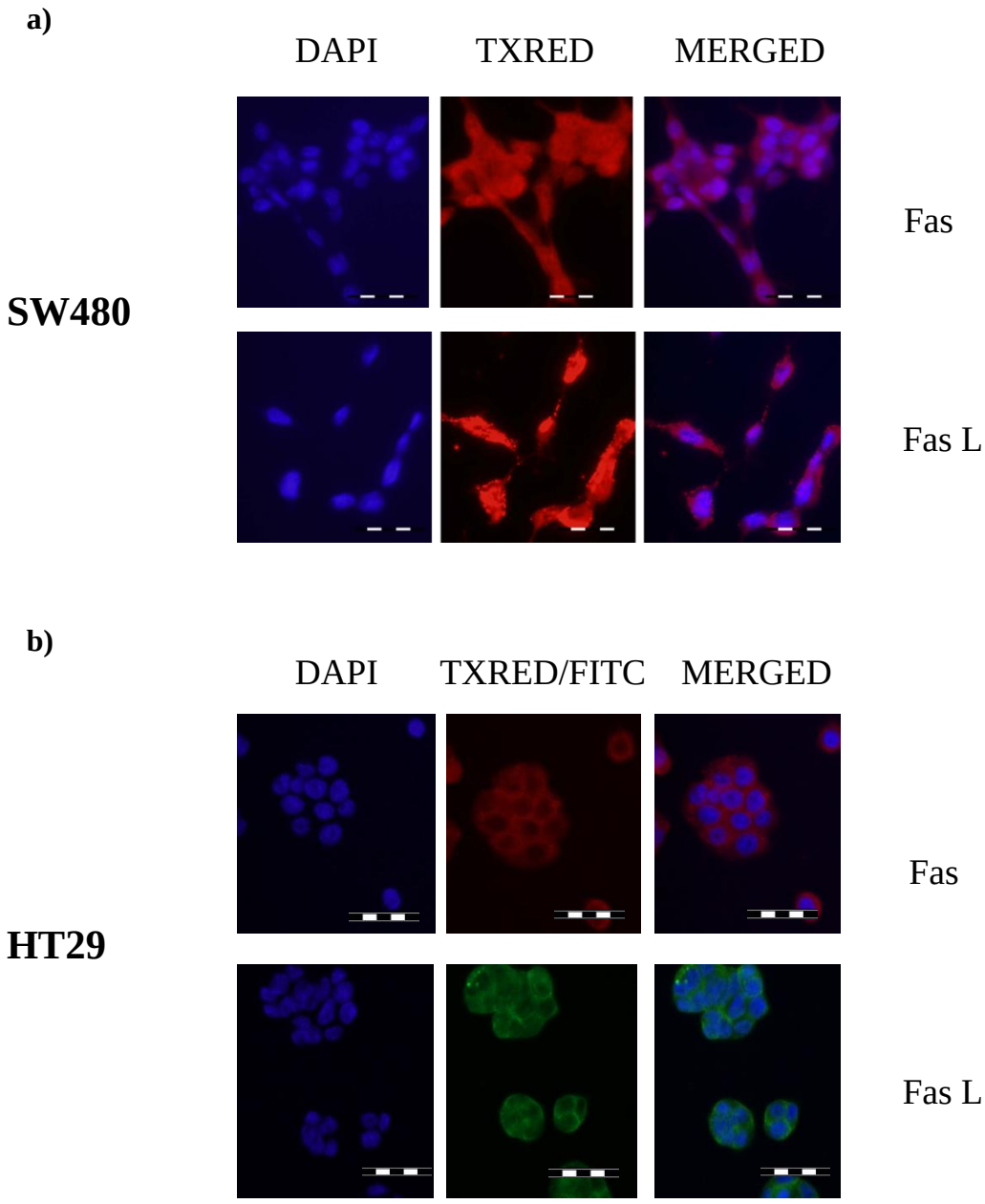


Figure 3.2.2.1 SW480 and HT29 cells express Fas and FasL. (a) SW480 cells or **(b)** HT29 cells were seeded at a concentration of $1x10^5$cells/ml onto sterile coverslips. 24 hours later, cells were fixed and immunofluorescently stained with anti-Fas or anti-FasL specific antibodies. Cells were counterstained with DAPI. Scale bar = 100μM.

3.2.3 Ligands for TLRs 4 and 5, but not TLR2 or TLR9, increase the expression of Fas and FasL in intestinal cancer cells

In order to investigate a role for TLRs in the expression of Fas and FasL, SW480 cells were treated with increasing concentrations of TLR ligands and changes in expression of Fas and FasL were examined (Figure 3.2.3.1a). Stimulation of cells with the TLR5 ligand, Flagellin, resulted in a significant increase in Fas ($p<0.01$) and FasL ($p<0.01$) mRNA expression (Figure 3.2.3.1a). This was confirmed at the protein level by Western blotting (Figure 3.2.3.1b) with densitometric analysis also showing a significant increase in both Fas ($p<0.01$) and FasL ($p<0.01$) (Figure 3.2.3.1c).

Stimulation of SW480 cells with the TLR4 ligand, LPS also leads to an increase in expression of Fas and FasL both at the mRNA (Figure 3.2.3.2a) and protein (Figure 3.2.3.2b) level. Densitometric analysis confirmed that the upregulation of Fas ($p<0.05$) and FasL ($p>0.01$) expression upon LPS stimulation was significant (Figure 3.2.3.2c).

In contrast, Pam3CSK4, a TLR2 ligand, had no effect on the expression level of Fas and FasL in SW480 cells (Figure 3.2.3.3a). In addition, treatment of SW480 cells with peptidoglycan (PGN), another TLR 2 ligand, failed to alter Fas expression (Figure 3.2.3.3b). Similarly, transfection of SW480 cells with CpGDNA, a TLR9 ligand, had no effect on Fas and FasL expression (Figure 3.2.3.3c).

In order to verify that SW480 cells were capable of responding to TLR2 and TLR9 ligands, SW480 cells were stimulated with Pam3CSK4 and CpGDNA and the expression levels of other proteins examined. Stimulation of SW480 cells with Pam3CSK4 increased the expression of the gap-junction protein, Connexin-43 (Cx43) (Figure 3.2.3.3d) while expression of the Wnt-signalling protein Frizzled5 (Fzd5) was increased by TLR9 stimulation (Figure 3.2.3.3e). These genes were selected as they have been previously shown to be activated by Pam3CSK4 and CpG DNA respectively, in intestinal cancer cells [185, 248]. Furthermore, despite failing to induce Fas and FasL in SW480 cells, peptidoglycan (PGN) induced a significant increase ($p<0.01$) in IL-8 secretion by these cells (Figure 3.2.3.3f).

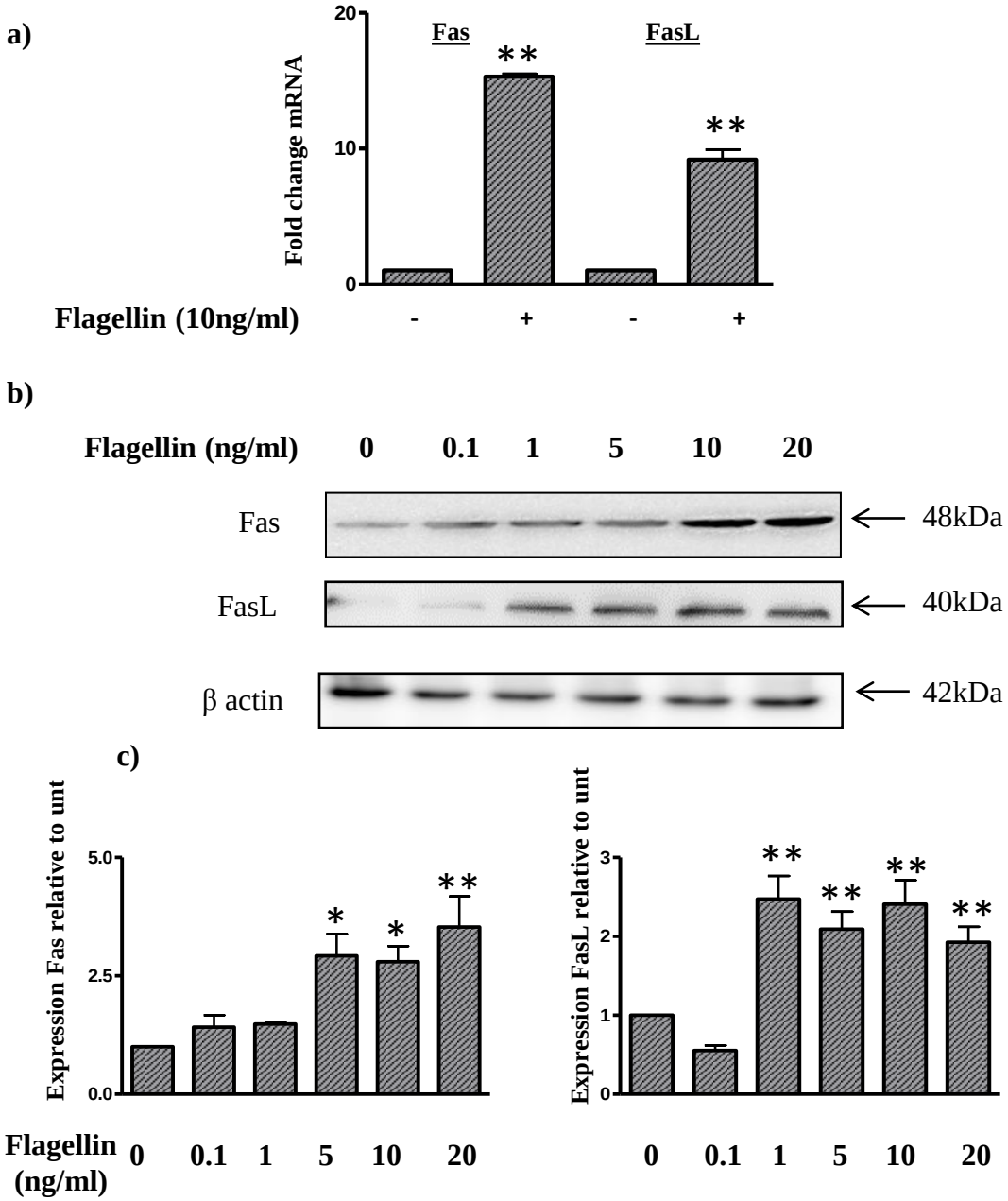


Figure 3.2.3.1 Stimulation of SW480 cells with Flagellin upregulates Fas and FasL expression. **(a)** SW480 cells were treated with increasing concentrations of Flagellin for 24 hrs. Total RNA was extracted and Fas and FasL mRNA levels were measured by real-time RT-PCR. **(b)** Cell lysates were separated by SDS-PAGE and probed with anti-Fas, anti-FasL or anti-β-actin specific antibodies.

(c) Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments. Values are plotted as Mean S.E.M. (**p<0.01, *p<0.05 as compared to untreated control; one way ANOVA, Tukeys post hoc t-test).

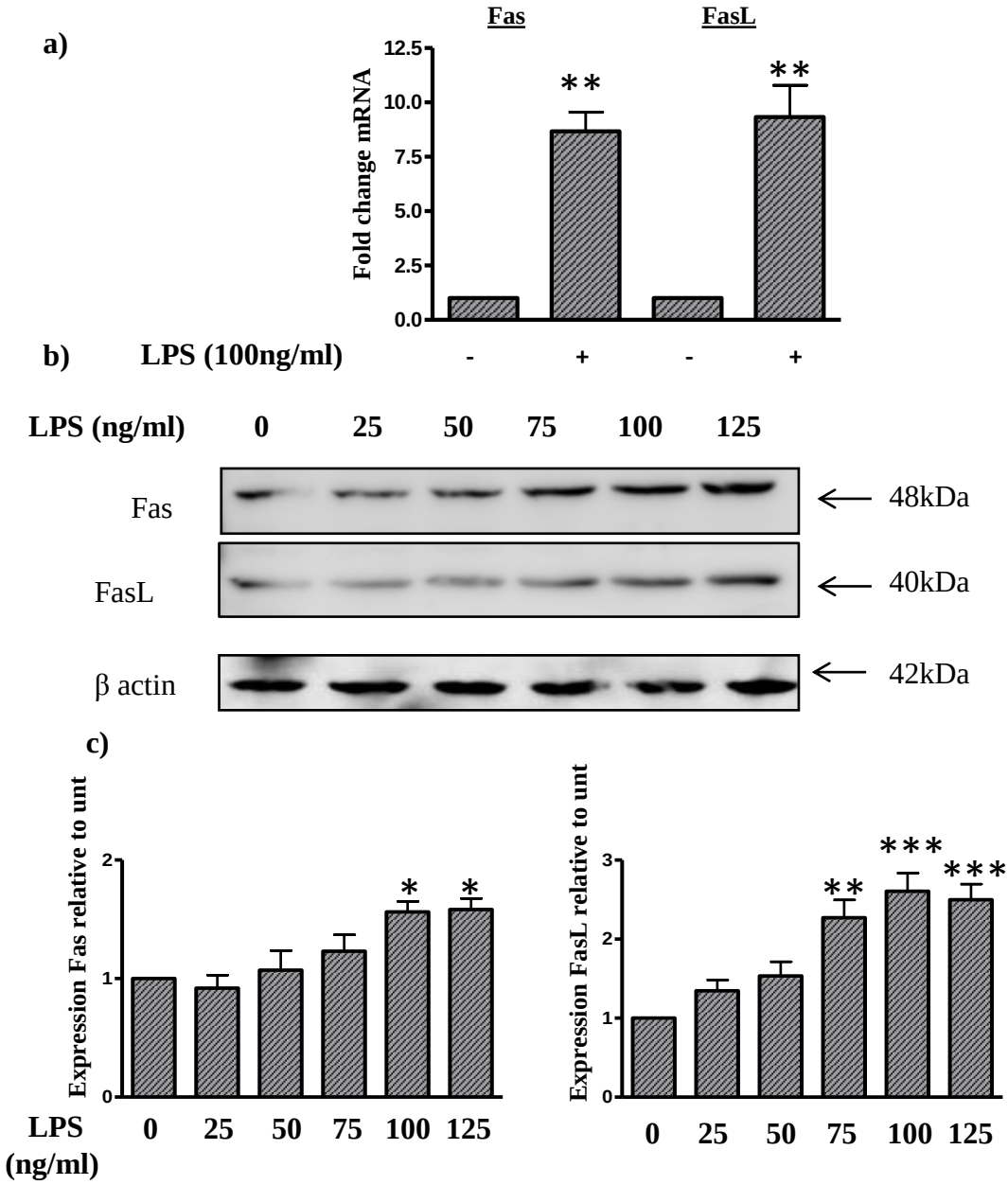


Figure 3.2.3.2 Stimulation of SW480 cells with LPS upregulates Fas and FasL expression. **(a)** SW480 cells were treated with increasing concentrations of LPS for 24 hrs. Total RNA was extracted and Fas and FasL mRNA levels were measured by real-time RT-PCR. **(b)** Cell lysates were separated by SDS-PAGE and probed with anti-Fas, anti-FasL or anti-β-actin specific antibodies. **(c)** Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments. Values are plotted as Mean S.E.M. (***$p>0.001$, **$p<0.01$, *$p<0.05$ as compared to untreated control; one way ANOVA, Tukeys post hoc t-test).

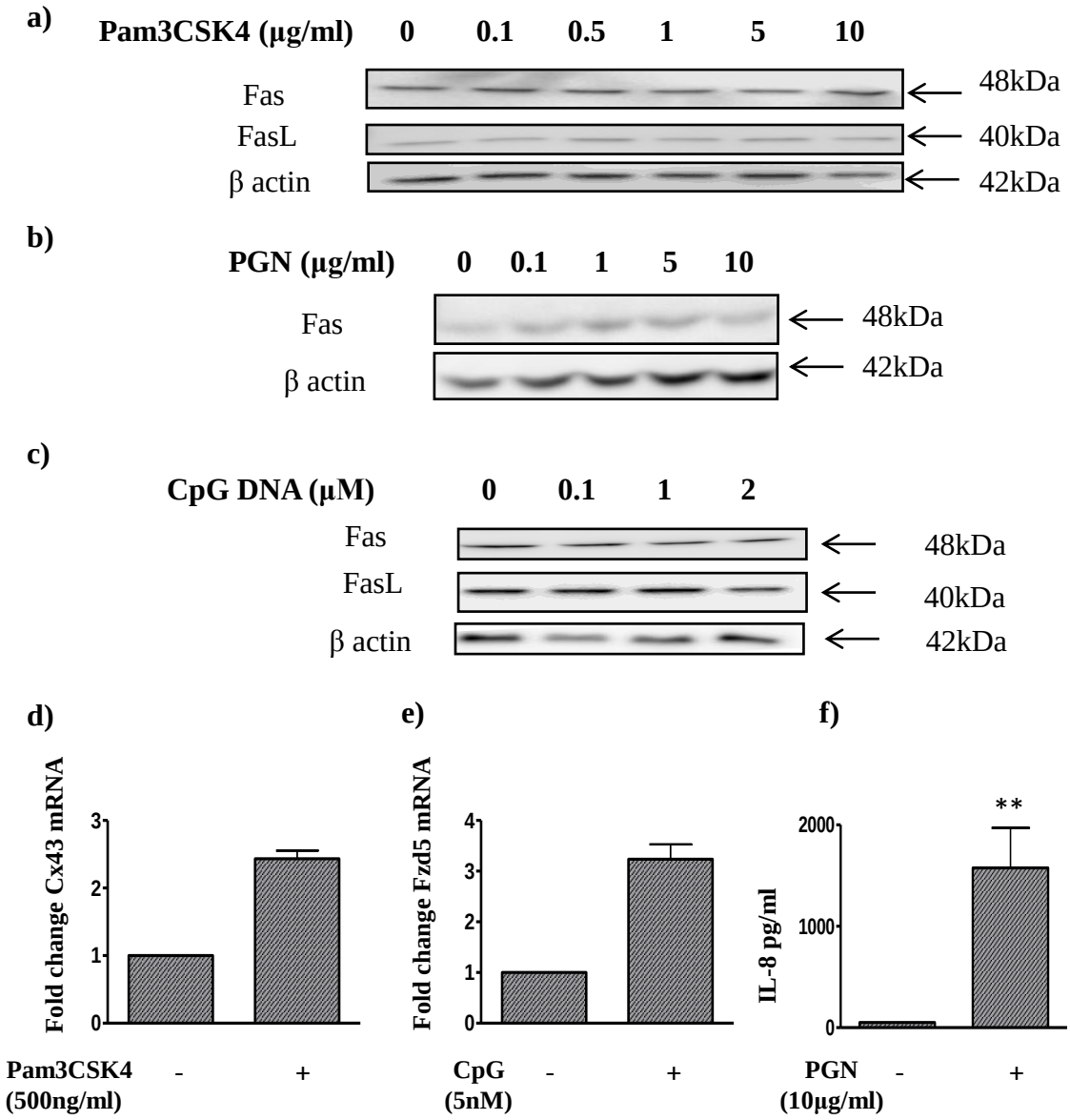


Figure 3.2.3.3 Fas and FasL expression in SW480 cells is unaffected by stimulation with TLR2 and TLR9 ligands. SW480 cells were treated with increasing concentrations of **(a)** Pam3CSK4 **(b)** PGN, and **(c)** CpG for 24 hrs. Cell lysates were separated by SDS-PAGE and probed with anti-Fas, anti-FasL or anti-β-actin specific antibodies. SW480 cells were treated with **(d)** Pam3CSK4 or **(e)** CpG DNA for 8 hrs, total RNA was extracted, and Cx43 and Fxd5 mRNA levels were measured by real-time RT-PCR. **(f)** SW480 cells were stimulated with PGN for 24 hrs and IL-8 levels measured by ELISA. Values are plotted as Mean S.E.M. (**$p<0.01$ as compared to untreated control; one way ANOVA, Tukeys post hoc t-test).

3.2.4 The increase in expression of Fas and FasL in intestinal cancer cells in response to Flagellin occurs in an NFκB-, and PI3Kinase -dependant manner.

TLR4 and TLR5 activate downstream signalling pathways such as the NFκB, the MAP kinase and the PI3K/Akt pathways. In order to investigate which pathway was responsible for the upregulation of Fas and FasL by Flagellin, SW480 cells were pre-treated with inhibitors of NF-κB, PI3K, or p38 MAP kinase signalling pathways prior to stimulation with Flagellin. TPCA-1, an IKKβ inhibitor, reduced Flagellin-induced upregulation of both Fas ($p<0.05$) and FasL ($p<0.05$) expression (Figure 3.2.4.1a and b). Pre-treatment of cells with LY294002, a PI3K inhibitor, also significantly prevented Flagellin-induced Fas ($p<0.001$) and FasL ($p<0.01$) expression (Figure 3.2.4.1a and c). In contrast, pre-treatment of SW480 cells with SB208530, a p38 MAPK inhibitor did not alter the ability of SW480 to upregulate Fas or FasL in response to Flagellin (Figure 3.2.4.1a).

3.2.5 The increase in expression of FasL on intestinal cancer cells in response to LPS occurs in a p38 MAP kinase-dependant manner.

Similarly, SW480 cells were pre-treated with inhibitors of NF-κB, PI3K, or p38 MAP kinase signalling pathway prior to stimulation with LPS. In contrast to the results seen with Flagellin, pre-treatment with the P13K inhibitor, LY294002 had no effect on the ability of LPS to upregulate FasL (Figure 3.2.5.1a and b). The p38 MAPK inhibitor, SB208530, prevented LPS-induced FasL upregulation ($p<0.01$) (Figure 3.2.5.1a and c) but did not prevent the induction of Fas by LPS. Pre-treatment of SW480 cells with TPCA-1 did not alter the ability of SW480 to upregulate Fas or FasL in response to LPS (Figure 3.2.5.1a).

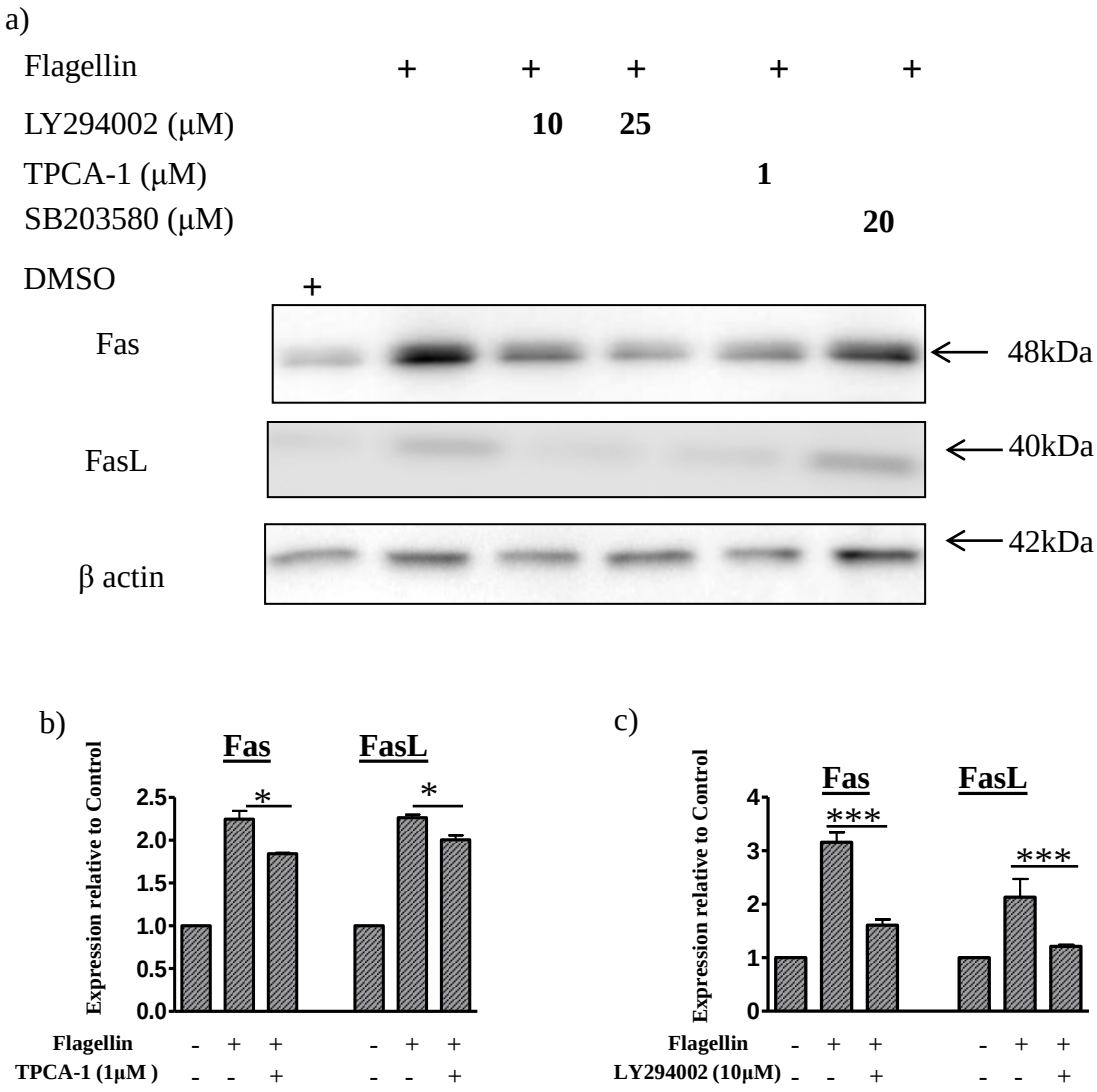


Figure 3.2.4.1 Pre-treatment with an NFκB inhibitor or a PI3K inhibitor prevents the upregulation of Fas and FasL by Flagellin. **(a)** SW480 cells were pre-treated with TPCA-1 or LY294002 for one hr prior to stimulation with 10ng/ml Flagellin for 24 hrs. Cell lysates were separated by SDS-PAGE and probed with anti-Fas, anti-FasL or anti-β-actin specific antibodies. Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments for **(b)** TPCA-1 and **(c)** LY294002. Values are plotted as Mean S.E.M. (***$p<0.001$, *$p<0.05$ as compared to untreated control; one way ANOVA, Tukeys post hoc t-test).

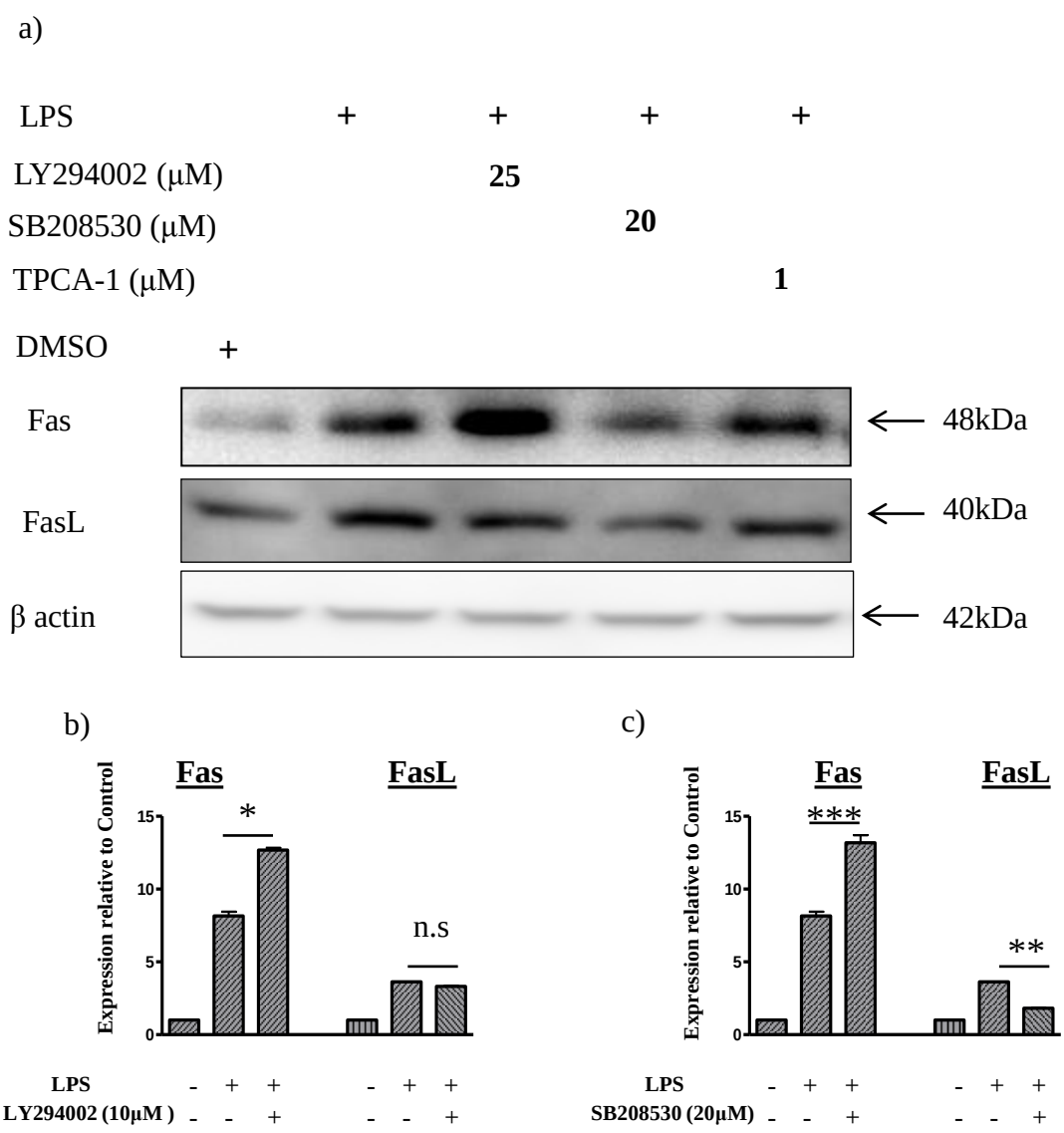


Figure 3.2.5.1 Pre-treatment with a PI3K inhibitor prevents the upregulation of Fas by LPS whilst pre-treatment with a p38 kinase inhibitor LPS-induced FasL expression. **(a)** SW480 cells were pre-treated with LY294002 or SB208530 for one hr prior to stimulation with 100ng/ml LPS for 24 hrs. Cell lysates were separated by SDS-PAGE and probed with anti-Fas, anti-FasL or anti-β-actin specific antibodies. Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments for **(b)** LY294002 and **(c)** SB208530. Values are plotted as Mean S.E.M. (***$p<0.001$, **$p<0.01$, **$p<0.05$ as compared to untreated control; one way ANOVA, Tukeys post hoc t-test).

3.2.6 Fas and FasL expression is reduced in the colons of germ-free, TLR4 and TLR5 knockout mice.

In order to determine the *in vivo* relevance of the induction of Fas and FasL expression by TLR4 and TLR5 ligands, colonic tissue from conventionally reared mice was assessed for Fas and FasL expression. Initially, a number of different concentrations of anti-Fas and anti-FasL antibodies were used in order to determine the optimum concentration to best visualise the expression levels of these proteins in the colon tissue (Figure 3.2.6.1 a and b). An isotype control was included in order to validate the specificity of the staining (Figure 3.2.6.1c).

Fas and FasL were found to be expressed throughout the colonic epithelia of conventionally reared mice, from the basal to the apical face, in a uniform manner (Figure 3.2.6.1 and b). In contrast, expression of Fas and FasL was reduced in the colonic epithelium of germ-free (GF) mice, as assessed by both immunohistochemistry (IHC) (Figure 3.2.6.2a) and Western blotting (Figure 3.2.6.2b and c), consistent with the lack of exposure of the intestinal cancer cells to commensal flora and thus TLR ligation. Conventionalisation of GF mice, however, restored Fas and FasL expression to levels to that seen in conventionally reared animals (Figure 3.2.6.2a), suggesting that Fas and FasL expression is, at least partially, dependent on colonisation of the colon by commensal bacteria.

Since both LPS and Flagellin upregulated Fas and FasL in intestinal cancer cells *in vitro*, the expression levels of Fas and FasL in the distal colon of TLR4 and TLR5 knock out (KO) mice were examined by IHC. In contrast to the immunohistochemical staining pattern observed in wild type (WT) mice, colonic tissue from both TLR4KO and TLR5KO mice demonstrated a marked reduction in expression of both Fas and FasL (Figure 3.2.6.3a). This reduction in expression of Fas and FasL in TLR4KO colonic tissue was confirmed by Western blotting (Figure 3.2.6.3b and c). Unfortunately, whole cell lysate from TLR5 tissue was not available for Western blotting. Consistent with the *in vitro* findings, Fas and FasL expression was unchanged in the IECs of TLR2KO mice, although it appeared somewhat reduced in the Western blot (Figure 3.2.6.3a and b). Whole cell lysate from colon tissue contains a mixed population of cells, including absorptive and secretory cells, as well as a plethora of cells of a lymphoid origin, each of which may vary in their

expression of Fas and FasL. Furthermore, it is impossible to quantify these different cell types within the lysate. In contrast, immunohistochemical staining and subsequent microscopy affords visualisation at the single cell level, allowing for cell type identification. This may explain the discrepancy between expression levels observed using the two techniques when comparing colonic tissue from WT and TLR2KO mice.

In order to investigate whether the reduction of expression of Fas and FasL in the colon of TLR4 and TLR5KO mice was specific, colon tissue was immunohistochemically stained for other proteins such as the Insulin Growth Receptor 1β, IGFR-1β. As can be seen in Figure 3.2.6.4a, IGFR-1β is uniformly expressed throughout the epithelial cells of the colon and this is unchanged in the colon tissue from TLR4 and TLR5KO mice. TNFR-1 is another member of the death receptor family and so its expression was also examined in the colon of TLR4 and TLR5KO mice relative to WT. Expression of TNFR-1 was found to be restricted to the apical surface of the colon in the wild type tissue and this pattern of expression was unchanged in the TLR4 and TLR5KO tissue (Figure 3.2.6.4b).

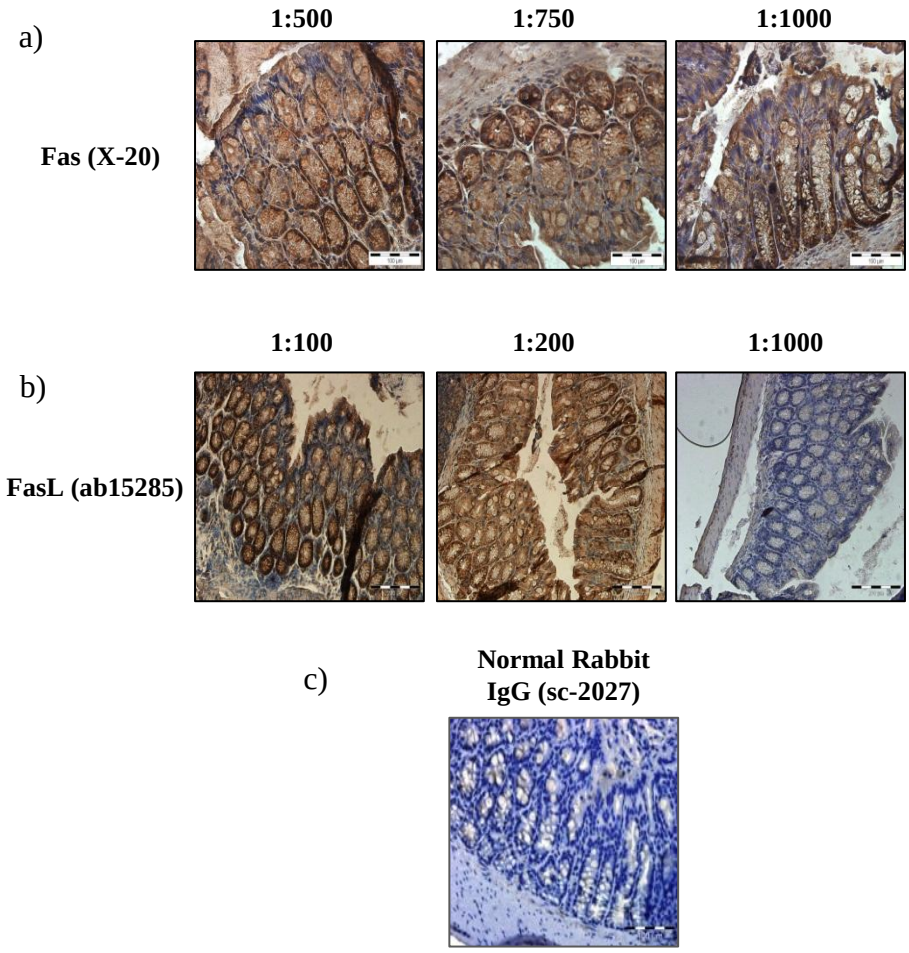


Figure 3.2.6.1 Optimisation of anti-Fas and anti-FasL antibodies for colon tissue immunohistochemistry. Paraffin-embedded colon tissue sections were rehydrated and heat-induced antigen retrieval performed. A range of dilutions of **(a)** anti-Fas (X20) and **(b)** FasL (ab15285) was used to immunohistochemically stain the tissue. Brown indicates positive staining. **(c)** Staining with a normal rabbit IgG isotype control, (sc-2027) was performed as a negative control. Scale bar = 100μM.

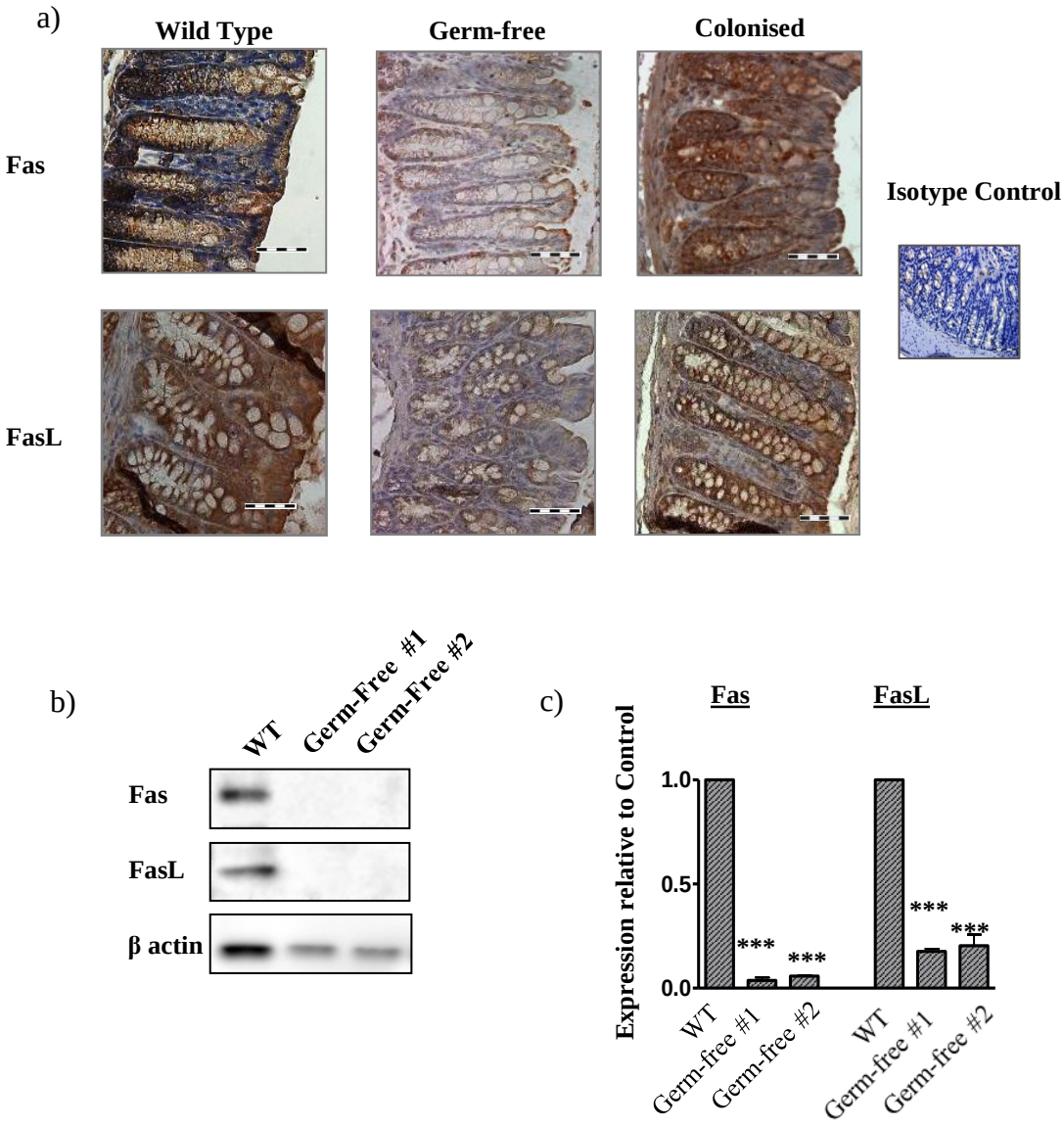


Figure 3.2.6.2 Expression of Fas and FasL is reduced in the colon tissue of germ-free mice relative to WT and conventionalised mice. (a) Paraffin-embedded colon tissue sections from WT, GF and GF-conventionalised for 49 days were rehydrated and immunohistochemically stained for Fas and FasL. Scale bar = 100μM. **(b)** Protein was extracted from colonic tissue and changes in Fas, FasL and β actin detected by Western blotting. **(c)** Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments. Values are plotted as Mean S.E.M. (***$p<0.001$, as compared to wildtype tissue, one way ANOVA, Tukeys post hoc t-test).

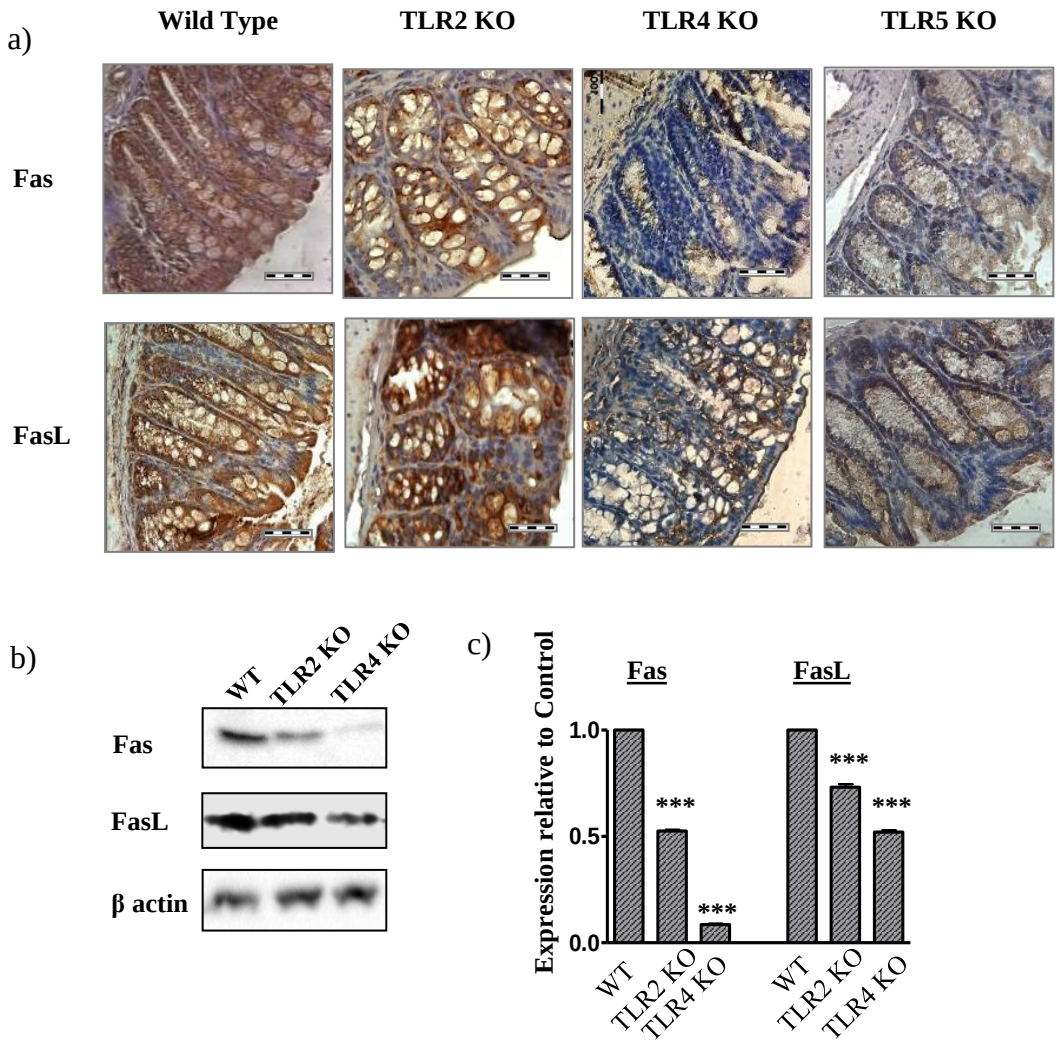


Figure 3.2.6.3 Expression of Fas and FasL is reduced in the colon tissue of TLR4 and TLR5KO mice relative to WT mice. (a) Paraffin-embedded colon tissue sections from WT, TLR2KO, TLR4KO and TLR5KO mice were rehydrated and immunohistochemically stained for Fas and FasL. Scale bar = 100μM. Data shown are representative of colonic tissue obtained from 5 mice per group. **(b)** Protein was extracted from colonic tissue and changes in Fas, FasL and β actin detected by Western blotting. Data shown are representative of tissue from 5 mice. **(c)** Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments. Values are plotted as Mean S.E.M. (***$p<0.001$, as compared to wildtype tissue, one way ANOVA, Tukeys post hoc t-test).

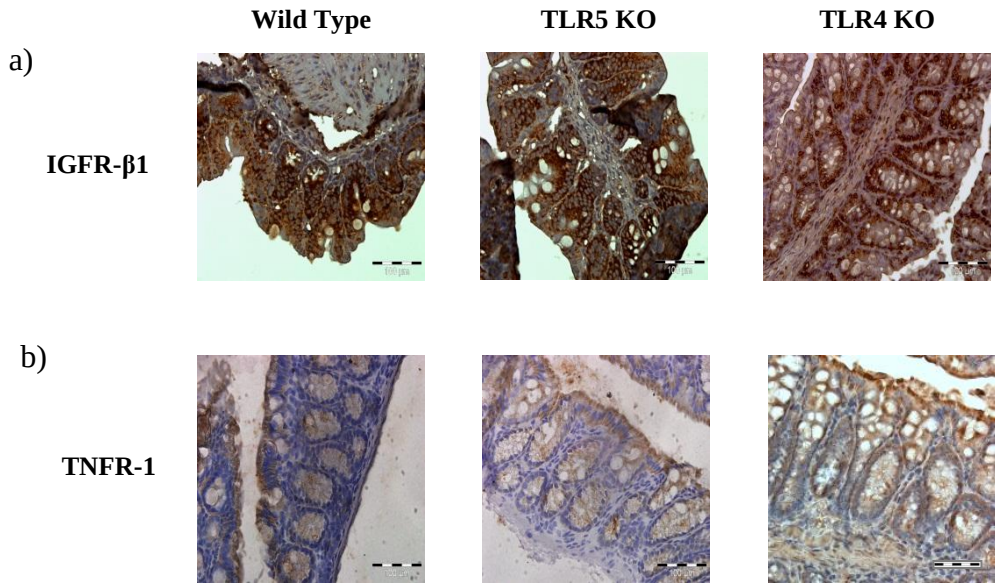


Figure 3.2.6.4 Expression of IGFR-β1 and TNFR-1 remains unchanged in the distal colon of TLR4 and TLR5KO mice relative to WT mice. Paraffin-embedded distal colon tissue sections from wildtype, TLR4 KO and TLR5 KO mice were rehydrated and immunohistochemically stained for **(a)** IGFR-β1 **or (b)** TNFR-1. Scale bar = 100μM.

3.2.7 Expression levels of Fas and FasL is altered in other tissues from TLR4 and TLR5KO mice.

In order to further investigate the possible extent of alterations in Fas and FasL expression in TLR 4 and 5KO mice, tissue was assessed from other organs. As can be seen in Figure 3.2.7.1a, Fas is expressed throughout the cytoplasm in the WT lung tissue, and this is greatly reduced in the TLR4 and TLR5KO tissue. FasL is also expressed throughout the cytoplasm of the lung epithelium and its expression is markedly reduced in the epithelium of the TLR5KO lung tissue. In the TLR4KO tissue, FasL expression appears to be completely abrogated. In the spleen, FasL is expressed in the dense white pulp and, to a lesser extent, in the surrounding fibrous red pulp (Figure 3.2.7.1b and d). Given the important role of FasL in immunity, this finding is in agreement with the literature as the white pulp is populated by T lymphocytes, which express high levels of FasL upon stimulation [249]. Expression levels of FasL in the TLR5 KO spleen tissue are reduced relative to the WT spleen. Fas is expressed primarily in the white pulp in the WT tissue [250] and this is also reduced in the TLR5KO tissue.

Since the tissues examined in this study to date were either highly immunogenic (i.e. the spleen) or colonised by commensal bacterial flora (i.e. the colon and the lung), I next examined expression levels of Fas and FasL in other tissues such as the kidney and the brain. FasL and Fas were found to be primarily expressed in the renal tubules of the kidney in WT tissue (Figure 3.2.7.2a and c), which is in agreement with the published literature [251]. Neither Fas nor FasL expression was affected upon TLR4 or TLR5KO in the kidney tissue. Neuronal cells of the cerebellum were also found to express relatively high levels of Fas and FasL and this was unchanged in TLR4 and TLR5KO mice (Figure 3.2.7.2b). These results suggest that the potential of TLR4 and TLR5 to regulate Fas and FasL expression may be tissue specific and/or dependant on the presence of commensal flora.

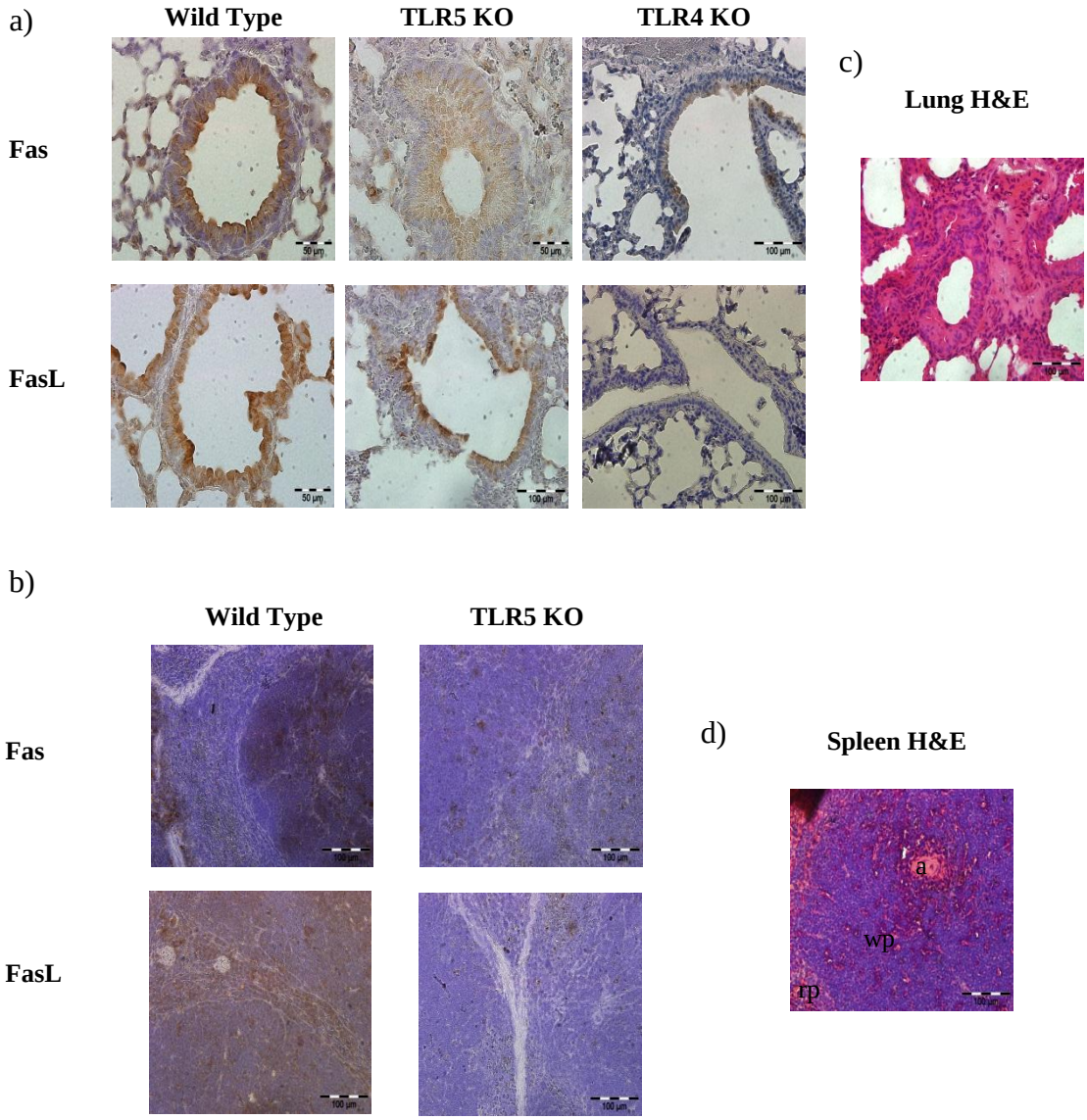


Figure 3.2.7.1 Expression of Fas and FasL is reduced in the lung epithelium and spleen of TLR4 and TLR5KO mice relative to WT mice. Paraffin-embedded **(a)** lung and **(b)** spleen tissue sections from WT, TLR4KO and TLR5KO mice were rehydrated and immunohistochemically stained for Fas and FasL. Standard H and E protocol was used to stain **(c)** lung and **(d)** spleen tissue, rp = red pulp; wp = white pulp; a = arteriole. Scale bar = 100μM.

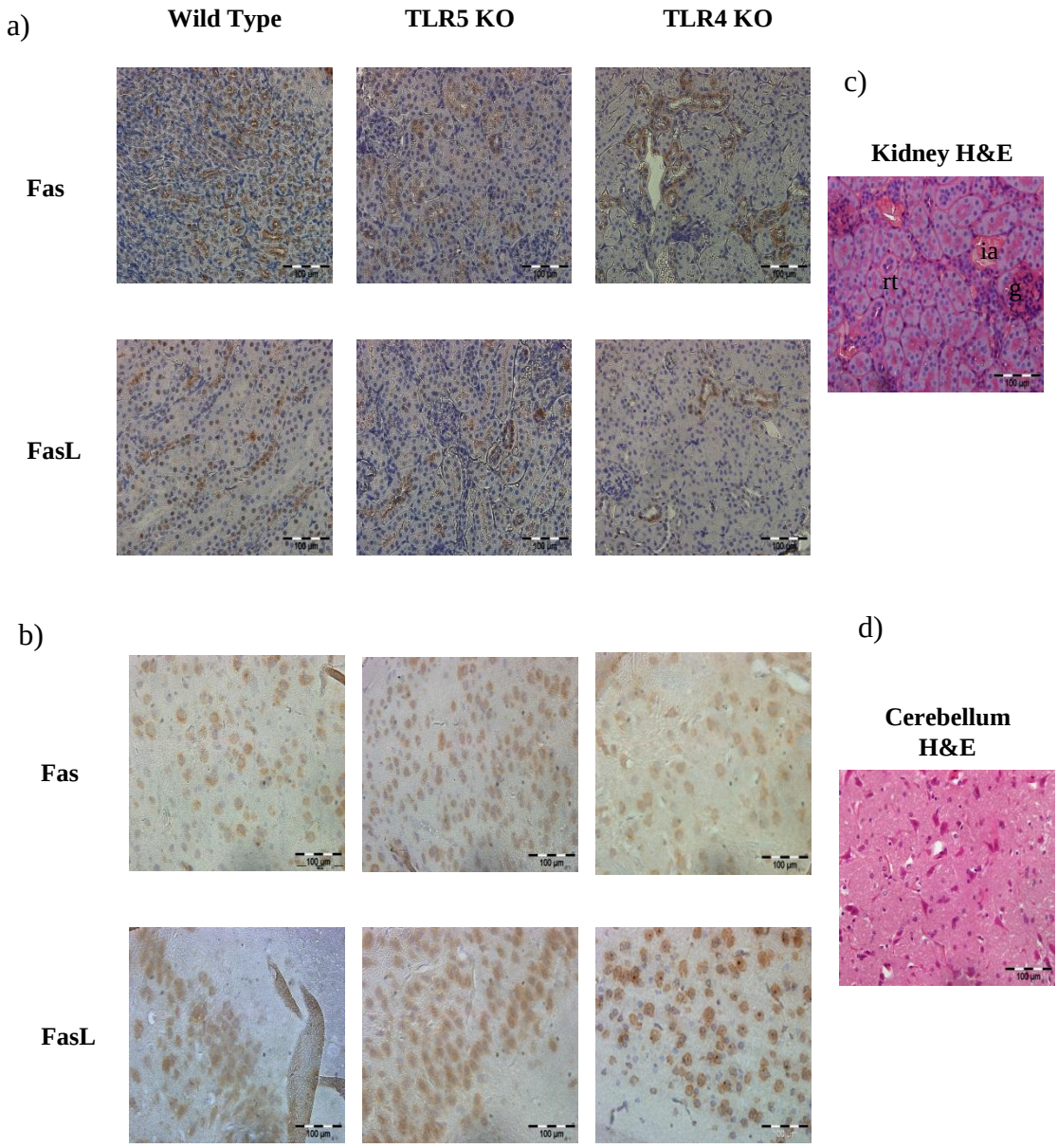


Figure 3.2.7.2 Expression of Fas and FasL remains unchanged in the kidney and cerebellum of TLR4 and TLR5KO mice relative to WT mice. Paraffin-embedded **(a)** kidney and **(b)** cerebellum tissue sections from wildtype, TLR4 KO and TLR5KO mice were rehydrated immunohistochemically stained for Fas and FasL. Standard H and E protocol was used to stain **(c)** kidney and **(d)** cerebellum tissue, g = glomerulus, rt = renal tubule; ia = interlobular artery. Scale bar = 100μM

3.2.8 The altered Fas and FasL expression in TLR4 and TLR5KO mice may be due to differences in adaptor proteins

The findings so far are suggestive of a level of specificity in the ability of TLR ligands to upregulate Fas and FasL in the intestine. One possible explanation for this specificity may be differences between the signalling pathways activated by the TLRs. Whilst all four TLRs examined in this study (TLR2, 4, 5 and 9) utilise the TLR adaptor protein, myeloid differentiation primary response gene (MyD88), only TLRs 4 [180] and 5 [252] utilise the adaptor molecule, TIR-domain-containing adapter-inducing interferon-β (TRIF). This utilisation of TRIF by TLR5 was shown specifically in intestinal cancer cells [253]. Therefore Fas and FasL expression in TRIF and MyD88 knockout tissue was examined. Expression of both Fas and FasL protein was significantly reduced in TRIF KO tissue, as assessed by both immunohistochemistry (Figure 3.2.8.1a) and Western blotting (Figure 3.2.8.1 b and c). Expression was also reduced, albeit to a lesser extent, in MyD88 KO tissue. These findings indicate that signalling through TRIF by ligands for TLRs 4 and 5, but not TLRs 2 or 9, may be responsible for the induction of Fas and FasL expression in intestinal cancer cells.

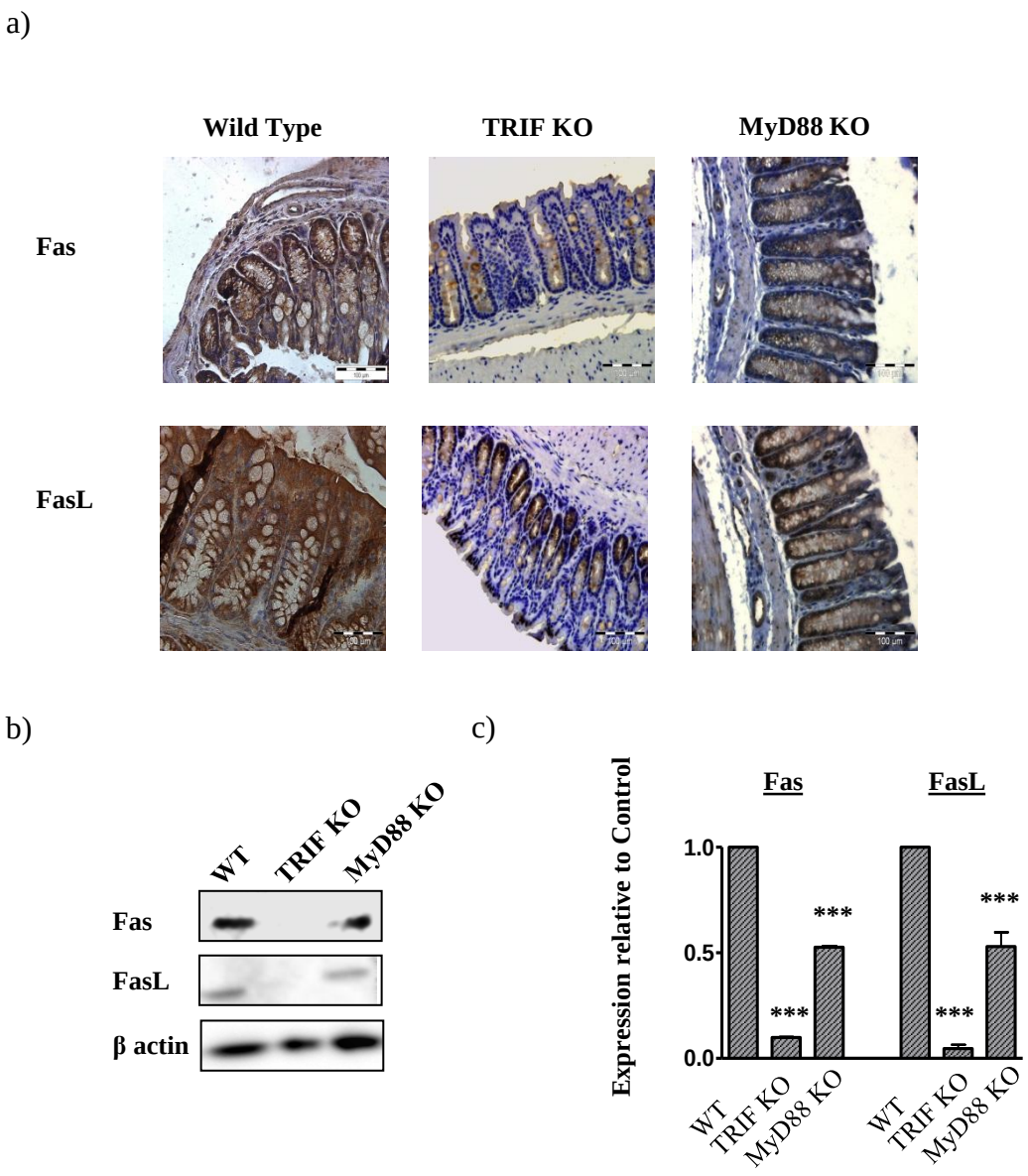


Figure 3.2.8.1. Expression of Fas and FasL is reduced in the distal colon of MyD88 and TRIF KO mice relative to WT mice. (a) Paraffin-embedded distal colon tissue sections from WT, TRIF KO and MyD88 KO mice were rehydrated and immunohistochemically stained for Fas and FasL. Scale bar = 100μM. **(b)** Protein was extracted from colonic tissue and changes in Fas, FasL and β actin detected by Western blotting. Data shown are representative of tissue from 5 mice. **(c)** Semi-quantitative analysis of Fas and FasL expression was determined by densitometry and normalised to β actin from 3 independent experiments *** $p<0.001$, Values are shown as Mean ± SEM.

3.3 Discussion

I have shown that stimulation through TLRs 4 and 5 either by their cognate ligands or by commensal flora increases expression of Fas and FasL *in vitro* and *in vivo* in tissues that are either immunogenic or are exposed to a commensal bacterial population, i.e. the colon, the lung and the spleen. It was also shown that this regulation of expression is specific to Fas and FasL and may be mediated by the TLR adapter protein, TRIF, in the colon.

The regulation of Fas and FasL has been most extensively studied in T cells of the adaptive immune system, where expression of both receptor and ligand are upregulated upon T cell receptor crosslinking [254]. Recently, studies have characterised the role of Fas and FasL in cells of the innate immune system and upregulation of Fas by TLR 2, 3, 4, 7 and 9 ligands has been reported in macrophages [244-246]. To the best of my knowledge, no report has demonstrated an upregulation of Fas and FasL by PAMPs in intestinal cancer cells. Treatment of SW480 cells with LPS and Flagellin led to an upregulation of Fas and FasL expression. To determine whether TLR4 and TLR5 were specific in their ability to upregulate Fas and FasL expression in intestinal cancer cells, SW480 cells were stimulated with ligands for a number of other TLRs including TLRs 2, 3 and 9. Despite being generally responsive to TLR2 and 9 ligands, the expression levels of Fas and FasL were unaltered upon Pam3CSK4 and CpG-ODN stimulation of SW480 cells. Thus ligands for TLRs 2, 4, 5 and 9 were used in further investigations. A recent study found that CpG-ODN is capable of downregulating FasL expression on HepG2 cells, a hepatocellular carcinoma cell line [255]. This group also found that treatment with CpG-ODN down-regulated the expression of Fas in human Jurkat cells. These observations are in contrast to the lack of response in intestinal cancer cells seen here. CpG-ODNs are short synthetic single-stranded DNA molecules possessing a partially or completely phosphorothioated (PS) backbone and contain unmethylated CpG motifs. There are three major classes of stimulatory CpG-ODNs which differ in their structure and ability to activate different cell types [256-258]. intestinal cancer cells have previously been shown to be responsive to CpG ODN 2006 [185], a class B CpG-ODN, which is the molecule used in this study to stimulate intestinal cancer cells. In contrast, Zheng., *et al* used the C-class CPG-

ODN M362 on liver-derived and immune cell lines [255]; differences which may explain the contrasting observations in terms of Fas and FasL expression.

TLRs are capable of initiating a wide variety of signalling cascades including the activation of NF-κB and of the p38 MAP kinase signalling pathways [259, 260]. It has been demonstrated here that the NF-κB and P13Kinase pathways are important for Flagellin-driven upregulation of Fas and FasL expression whilst FasL expression regulation initiated by LPS stimulation involves the activation of the p38 MAPK pathway. The FasL promoter region has been shown to have two distinct NFκB binding sites and NFκB has been shown to directly regulate FasL expression [35]. Recently, 3 NFκB sites have also been found in the Fas promoter region which, when methylated, lead to the reduced binding of this transcription factor [261]. Additionally, NF-κB has been implicated in the induction of Fas expression by TNF-α stimulation [31, 262]. The PI3K pathway also has been shown to be important in the regulation of FasL expression. For example, phosphorylation of MADS box transcription enhancer factor 2 by Extracellular-signal-regulated kinase 5 enhances its transcriptional activity downstream of PI3K activation, although in fibroblasts this led to a downregulation of FasL expression [263]. Furthermore, consistent with the data presented here demonstrating that the p38 kinase inhibitor, SB208530, prevented LPS-induced FasL expression in intestinal cancer cells, transcription factors important for FasL expression have been shown to be phosphorylated and subsequently activated by p38 [264-266]. Thus, a number of diverse signalling pathways upstream of a myriad of transcription factors are responsible for the regulation of Fas and FasL expression, some of which may be cell or tissue-specific. My results indicated that in intestinal cancer cells, multiple pathways exist that are capable of regulating Fas and FasL expression.

GF mice showed markedly reduced Fas and FasL in the distal colon which supports the hypothesis that TLR signalling regulates the expression of Fas and FasL in the colon. Conventionalisation for 49 days however effectively reversed or ‘rescued’ the expression levels back to those seen in WT tissue suggesting that pathogen associated molecular pattern (PAMP) signalling through TLRs is responsible for this alteration.

Consistent with this, Fas and FasL expression was also reduced in the epithelial cells of the colon of TLR4 and TLR5KO mice. Since there was no change in TLR2 knockout mice, this suggests a level of specificity to the ability of TLRs to regulate Fas and FasL expression in the colon. These results are in contrast to a report that has shown that TLR2 is capable of increasing Fas expression on macrophages [246], which serves to again indicate the importance of cell lineage for this phenomena.

The lung epithelia, like the colon, forms a mucosal surface primed for pathogen entry by both expressing PRRs, and being constantly exposed to PAMPS [267]. Furthermore, the lung also has a commensal microbiota with which it interacts for homeostatic and protective purposes [268]. Consistent with this, a reduction in expression of Fas and FasL was evident in lung tissue from TLR4 and TLR5KO mice relative to WT mice.

Within the intestine, immune cells are distributed amongst the GALT. The M cells contained in such tissue constantly sample the luminal microenvironment of the gut. A recent study has shown that antigens administered orally via the bacterial poly-component vaccine Immunovac-VP-4, which contains a wide panel of PAMPs not only led to an increased population of rapid effector cells of mucosal immunity in GALT [269] but also a concurrent increase in the spleen, the organ in which they are activated [270, 271]. These findings therefore confirm that there is an interchange of lymphocytes between the GALT and the spleen. Furthermore, it is thought that dendritic cells present in the lamina propria are actually able to open the tight junctions between epithelial cells in order to send dendrites outside the epithelium and therefore are able to directly sample bacteria [272]. Being migratory, DCs can then transport pathogens to the mesenteric lymph node and the spleen for the induction of systemic immune responses. The trafficking of immune cells from mucosal surfaces is therefore a likely way in which PAMPs are able to alter expression levels of Fas and FasL in resident cell populations of the spleen.

Unlike the lung or colon, the kidney is rarely exposed to bacterial or viral PAMPs and although TLRs are expressed by renal cells, their activation remains largely limited to the action of danger associated molecular pattern (DAMPS) rather than exogenous PAMPS. Indeed, it is the high levels of endogenous TLR ligands such as High-mobility group box 1 (HMGB1) reported in the injured kidney that are likely to

be responsible for chronic kidney disease [273, 274]. In this study, kidney tissue from TLR4 and TLR5 KO mice showed unaltered expression levels of Fas and FasL consistent with the kidney being a relatively sterile environment.

The blood brain barrier (BBB), the major structure responsible for transporting molecules and cells selectively into the central nervous system (CNS), maintains relative immune deficiency inside this compartment [275]. Furthermore, endothelial cells of the BBB express FasL [276], which significantly limits the extravasation of Fas-bearing inflammatory cells thus suppressing the immune response in the CNS. Given this immune privilege, it is likely that the expression of Fas and FasL is regulated in the brain by means other than TLR ligation which would explain why expression was unchanged in brain tissue from TLR4 and TLR5KO mice relative to WT.

The differential use of TLR adaptor proteins helps to explain the distinct pathways activated by TLRs and this, too, is likely to be tissue specific. Upon TLR ligation, signalling cascades are activated via Toll/IL-1 receptor (TIR) domain-containing adaptors, such as MyD88, TIR-domain containing adapter protein (TIRAP), and Mal so eliciting inflammatory responses. It is well known that TLR4 utilizes a combination of both Mal/TIRAP-MyD88 (MyD88-dependent pathway) and TRAM-TRIF (MyD88-independent pathway) to transduce its signal. Recently, TLR5 has also been shown to utilise TRIF in intestinal cancer cells [277]. Interestingly, this group showed that impaired TRIF expression reduced TLR5-induced NFκB and MAPK activation in response to Flagellin, an observation that supports the finding here that inhibition of NFκB prevented Flagellin induced upregulation of Fas and FasL. Convincingly, expression of Fas and FasL was greatly reduced in TRIF KO mice, with only a modest reduction observed in MyD88 KO mice. In contrast, neither TLR2 nor TLR9 have been reported to use TRIF, consistent with a failure of TLR2 and TLR9 ligands to upregulate Fas and FasL. Furthermore, a recent study examining the respective importance of TRIF-dependant versus MyD88-dependant gene expression in intestinal cancer cells revealed that the number of TRIF-dependent genes far exceeded the number of genes regulated by MyD88 [278]. This suggests that the specificity of TLR-induced upregulation of Fas and FasL in intestinal cancer cells may be due to the ability of TLRs 4 and 5 to utilise TRIF.

In conclusion, this chapter has contributed evidence for an unappreciated role of TLRs 4 and 5 in the regulation of Fas and FasL expression in the intestine. These finding are significant as they demonstrate a hitherto unknown link between Fas and FasL expression and the presence of commensal flora in the intestine.

Chapter 4

Fas as a modulator of intestinal TLR-mediated inflammation.

4.1 Introduction

Communication between the normal gut flora and the underlying intestinal epithelium has an essential role in promoting homeostasis and epithelial integrity in the intestinal mucosa. Intestinal epithelial cells (IECs) express a comprehensive panel of pathogen recognition receptors (PRRs) by which they are able to respond to luminal bacteria. These include the toll-like receptors (TLRs), nucleotide-binding oligomerization domain-like (NOD-like) receptors, the retinoic acid-inducible gene 1-like (RIG-I) receptors, the RNA helicases and the C-type Lectins. The recognition of pathogen activated molecular patterns (PAMPs) by TLRs on the cell surface or the endosomal surface of intestinal cancer cells leads to a swift immunological response via the production of pro-inflammatory and immune molecules produced in a variety of different ways such as the MAPK and p38 ERKs signalling pathways and the activation of transcription factors such as NFκB. For example, studies have shown that Flagellin activates TLR5 on intestinal cancer cells leading to the secretion of a number of pro-inflammatory cytokines and chemokines from intestinal cancer cells such as IL-8 and MIP3α. These are important in driving both the innate [188] and adaptive immune responses in the colon to enteropathogenic organisms [189]. Similarly, despite generally having relatively low expression levels of TLR4, intestinal cancer cells have been found to have a functionally active response to LPS resulting in NFκB activation and resulting in the induction of pro inflammatory cytokines [178, 180]. In this way, the detection of PAMPs by PRRs is thought to lead to clearance of gut infecting pathogens and the resolution of infection. If pathogens cannot be eliminated, chronic inflammation may occur and aberrant PRR signalling is thought to be a central contributor to the pathophysiology of inflammatory bowel disease (IBD). Indeed genetic-association studies have demonstrated a link between PRR genes, including *NOD2*, *NLRP3* and a number of TLR genes with IBD susceptibility [235, 279].

Signals from commensal bacteria are also mediated via intestinal cancer cell PRRs and they have been shown to contribute to epithelial homeostasis and repair [228]. Mice that are deficient in the TLR adaptor protein, MyD88, exhibit enhanced apoptosis of epithelial cells and diminished epithelial cell proliferation in response to dextran sodium sulphate (DSS) relative to DSS-treated wild-type (WT) mice [280], indicating that signalling through MyD88 in IECs is protective in the colon.

Similarly, loss of TLR2 has been shown to lead to an exacerbation of intestinal inflammation in DSS colitis which is associated with high morbidity and mortality [281]. Intestinal cancer cell-derived PRR signalling has been shown to lead to the expression of cytoprotective heat-shock proteins, epidermal growth factor receptor ligands [228] [282], and Trefoil Factor 3 [283] as well as promoting tight junction formation [192, 284], all of which are important in epithelial integrity and homeostasis. Microbial sensing by PRRs on IECs also drives the recruitment of stromal and myeloid cells to the intestinal mucosa, thereby facilitating regeneration of the epithelium after injury [285, 286]. Studies have also shown that the intestinal microbiota have a considerable effect on the development of the gastrointestinal tract. Germ-free (GF) mice have a reduced intestinal surface area in comparison to conventional mice; are impaired in brush border differentiation, and have reduced villus thickness [287]. These defects have been attributed to the reduced cell regeneration and a longer cell cycle of the crypt cells in GF mice relative to conventionalised animals [288]. Furthermore, new-born mammals have been found to have a functionally and structurally immature gastrointestinal (GI) tract that is only matured upon colonisation [289]. These data indicate that bacterial signalling through PRRs is essential for GI development and homeostasis.

Given the constant presence of the commensal flora, intestinal cancer cells must therefore be maintained in a state of immune tolerance. The expression of negative regulators of PRR signalling such as single Ig IL-1R-related molecule (SIGIRR) (also known as Tir8) and Toll interacting protein (TOLLIP), peroxisome proliferator-activated receptor-γ (PPARγ) and interleukin-1 receptor-associated kinase monocytes/macrophages (IRAKM) by intestinal cancer cells, is critical in controlling the homeostasis and innate immune responses of the colon to enteric microflora. Commensal bacteria have been shown to upregulate the expression of TLR inhibitory proteins on intestinal cancer cells and intestinal cancer cells deficient in SIGIRR are more susceptible to commensal-dependent intestinal inflammation [290]. This indicates that the intrinsic expression of TLR inhibitory proteins by intestinal cancer cells in response to commensal bacteria regulates the communication between commensal bacteria and the host immune system [291].

Fas (APO-1/CD95) is a membrane receptor also expressed in the intestinal epithelium and studies have shown that Fas is constitutively expressed within the

cytoplasm and at the basolateral surface of every colonic epithelial cell [238] irrespective of location. Engagement of Fas with its ligand, FasL, has been well characterised in terms of apoptosis. Fas associated death domain, FADD, is recruited to the receptor and the death inducing signalling complex (DISC) is formed. The DISC activates the initiator protease, caspase-8, which triggers activation of the effector proteases, caspase-3 and -7, that drive the cellular changes characteristic of apoptosis. Since expression of FasL is highly limited in the intestine [238], the Fas/FasL system is unlikely to play a role in epithelial regeneration. In ulcerative colitis (UC), although the number of Fas-positive cells in the intestine is increased, investigations have shown that Fas-mediated apoptosis is inhibited rather than increased, and it is now the augmented immune cell infiltrate that is considered to be the major cause of the clinical characteristics of IBD [123, 292].

In contrast to its role in mediating cell death, Fas has been shown to also activate non-apoptotic signalling pathways in a number of different cell types including macrophages, neuronal cells and intestinal cancer cells [101, 104, 221] with Fas ligation shown to induce the production of a number of pro inflammatory cytokines including IL-8, IL-6 and MCP-1 in intestinal cancer cells [221, 293]. The role of Fas in inflammation has also been assessed using *lpr* mice, which lack a functional Fas receptor and thus cannot be activated by FasL. In models of pulmonary inflammation, *lpr* mice exhibit reduced cytokine secretion and neutrophil influx, together with a reduction in epithelial cell apoptosis and tissue damage [117] [118].

Although Fas has been reported to directly activate key transcription factors in order to induce the secretion of pro-inflammatory cytokines [218-221], several lines of evidence suggest a level of crosstalk exists between the Fas and the TLR signalling pathways [104, 108, 222, 223]. Peritoneal macrophages from *lpr* or *gld* (FasL deficient) mice have been shown to have a diminished ability to produce IL-6 in response to LPS [104]. These *lpr* mice exhibit suppressed LPS and IL-1 induced NF-κB activation and cytokine expression suggesting that Fas ligation enhances IL-R1/TLR4 signalling to promote macrophage-mediated inflammation. Furthermore, interruption of Fas ligation was shown to suppress IL-1R1 and TLR4-induced IκBlα degradation in primary macrophages [104], thus suggesting that Fas ligation is able to modulate macrophage cytokine production by activation of NFκB.

Cross talk between Fas and TLR signalling has also recently been investigated in alveolar epithelial cells where investigators determined that Fas-induced inflammation occurred in a MyD88-dependant manner [222]. However, to the best of my knowledge, no study has directly investigated the crosstalk between these pathways in intestinal cancer cells.

AIM: The aim of this chapter was to determine if crosstalk exists between the Fas and TLR signalling pathways in intestinal cancer cells.

4.2 Results

4.2.1 Stimulation with agonistic Fas antibody and TLR4 or TLR5 ligands alters intestinal cancer cell-mediated cytokine production.

In the previous chapter, I demonstrated that TLR4 and 5 activation leads to the upregulation of expression of both Fas and FasL in intestinal cancer cells. As both TLR and Fas receptor engagement have been shown to independently induce the production of inflammatory cytokines in intestinal cancer cells, I next examined the response of these cells to simultaneous stimulation of Fas and TLR4 or TLR5. SW480 cells were pre-treated with the agonistic Fas antibody, CH-11, prior to stimulation with either LPS or Flagellin. Cytokine gene expression was initially analysed by qRT-PCR.

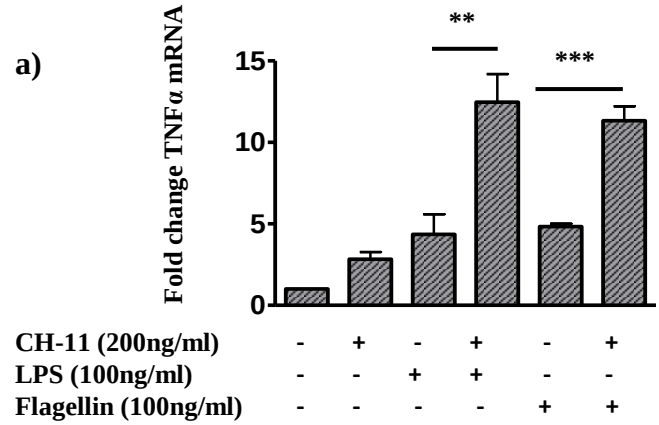
Treatment of SW480 cells with CH-11 resulted in a ~ 3 fold increase in TNFα production over untreated levels. Whilst LPS treatment led to a ~5 fold induction in TNFα transcription, stimulation of SW480 cells with CH-11 followed LPS resulted in a greater fold TNFα induction relative to LPS alone ($p<0.01$) (Figure 4.2.1.1a). Flagellin also induced a ~5 fold increase in TNFα production in SW480 cells and transcription of TNFα was augmented when cells were pre-treated with CH-11 followed by Flagellin treatment, relative to Flagellin treatment alone ($p>0.001$). Treatment of SW480 cells with CH-11 resulted in a ~5 fold increase in IL-8 relative to untreated levels (Figure 4.2.1.1b). Whilst LPS treatment led to a ~8 fold induction in IL-8 transcription, stimulation of SW480 cells with CH-11 followed by LPS resulted in a greater fold increase in IL-8 production relative to LPS alone ($p<0.001$). Similarly, whilst a three-fold increase in IL-8 was seen upon Flagellin treatment, stimulation with CH-11 with subsequent Flagellin stimulation augmented IL-8 transcription levels relative to Flagellin stimulation alone ($p<0.05$).

The augmented production of IL-8 in SW480 cells was confirmed by ELISA (Figure 4.2.1.2). In contrast to the mRNA data, CH-11 stimulation alone failed to induce IL-8 protein secretion in SW480 cells over 3 separate experiments, indicating a possible post-transcriptional level of regulation of this cytokine. LPS stimulation led to a 175pg/ml production of IL-8, whilst pre-stimulation with CH-11 augmented this to 240pg/ml, a statistically significant increase ($p<0.05$) relative to LPS alone.

Flagellin stimulation resulted in a modest increase in basal IL-8 secretion levels although these were also augmented upon CH-11 pre-treatment. To confirm that the augmented IL-8 production observed upon Fas and TLR ligation was not specific to SW480 cells, HT29 and HCT116 intestinal cancer cells were treated in the same manner. In contrast to the limited response of SW480 cells to CH-11, IL-8 production was increased by CH-11 alone in both HT29 and HCT116 cell lines to ~260 and ~150 pg/ml respectively (Figure 4.2.1.3a). Pre-treatment with CH-11 followed by LPS led to a three-fold increase in IL-8 protein production relative to LPS alone ($p<0.05$). Similarly, CH-11 pre-treatment followed by Flagellin stimulation resulted in a similar 3.5 fold increase in IL-8 production over Flagellin alone ($p<0.05$). Like HT29 cells, HCT116 cells doubled their basal expression of IL-8 in response to CH-11 (Figure 4.2.1.3b) and although LPS or Flagellin treatment alone failed to induce expression of IL-8 over untreated levels, pre-treatment with CH-11 led to a five-fold increase in IL-8 cells over Flagellin treatment alone ($p<0.001$).

4.2.2 Stimulation of SW480 cells with agonistic Fas antibody and *E. coli K12* augments cytokine production.

In order to investigate the physiological significance of the augmented TLR-induced cytokine production upon Fas stimulation, SW480 cells were pre-stimulated with CH-11 prior to infection with the gram negative bacterium, *E.coli* strain K12, which would be expected to stimulate cells through TLR4. Pre-stimulation with CH-11 followed by *E.coli K12* infection increased TNFα mRNA levels relative to *E.coli K12* infection alone ($p<0.001$) (Figure 4.2.2.1a). IL-8 mRNA production was also augmented with CH-11 pre-stimulation relative to *E. coli K12* treatment alone (Figure 4.2.2.1b), indicating that Fas receptor ligation modulates IL-8 and TNFα production in response to E. *coli K12* in intestinal cancer cells.



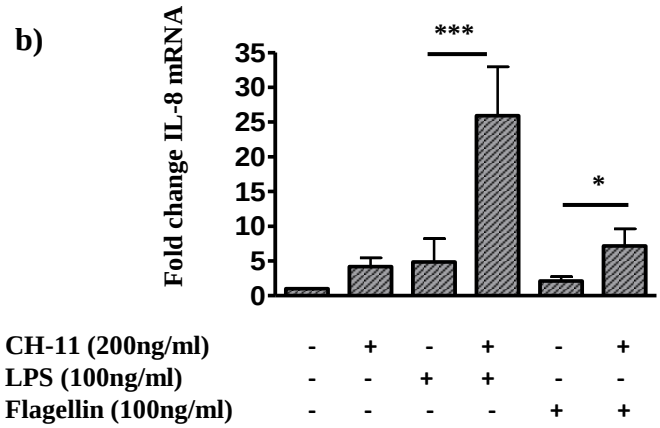


Figure 4.2.1.1 Pre-treatment of SW480 cells with CH-11 and TLR4 or TLR5 ligands modulates cytokine production. (a&b) SW480 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml Flagellin for 8 hrs. Changes in TNFα and IL-8 were detected by qRT-PCR. Statistical analysis was performed and statistical change determined comparing either LPS or Flagellin stimulation alone to LPS+CH-11 or Flagellin+CH-11. Values are plotted as Mean ± S.E.M. n=3. ***p<0.001, **p<0.01, *p<0.05; one way ANOVA, Tukeys post hoc t-test.

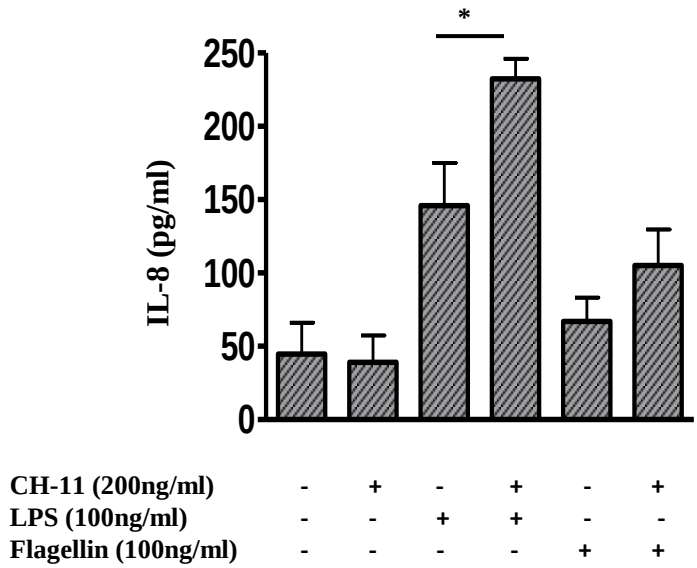


Figure 4.2.1.2 Pre-treatment of SW480 cells with CH-11 and TLR4 or TLR5 ligands augments IL-8 protein production. SW480 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml flagellin for 24 hrs. Supernatents were harvested and IL-8 protein concentration determined by ELISA. Statistical analysis was performed and statistical change determined comparing either LPS or Flagellin stimulation alone to LPS+CH-11 or Flagellin+CH-11. Values are plotted as Mean ± S.E.M. n=3. *$p<0.05$; one way ANOVA, Tukeys post hoc t-test.

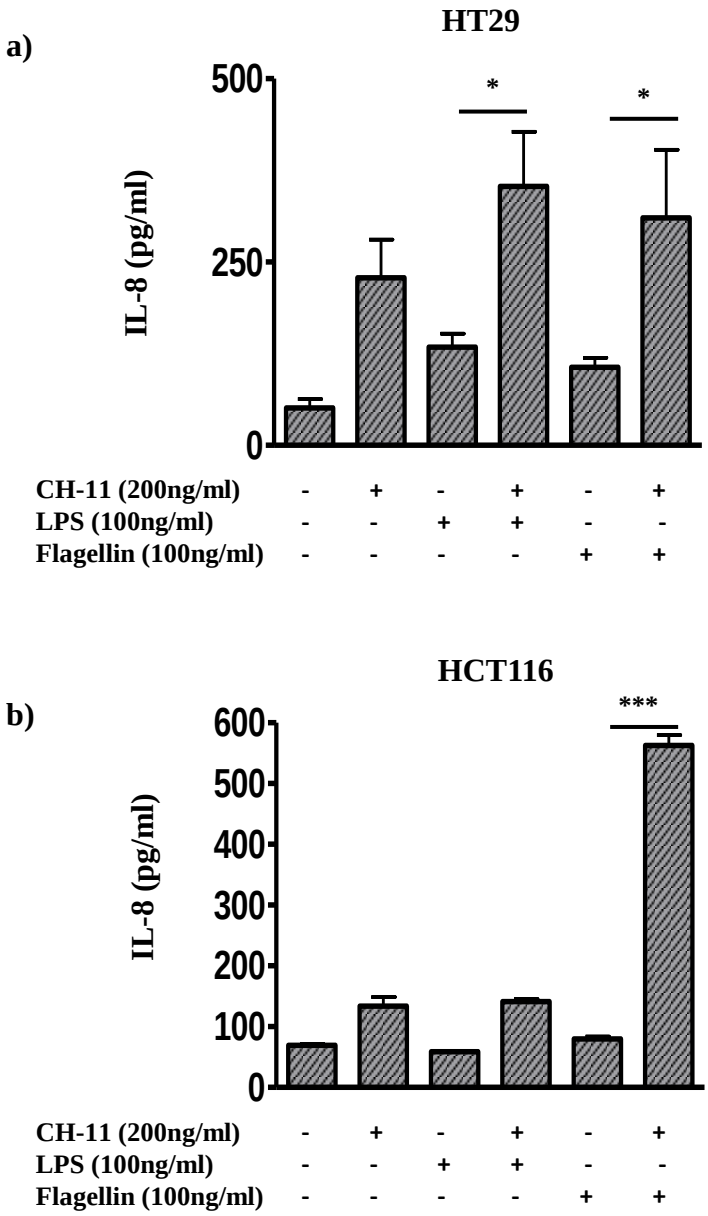
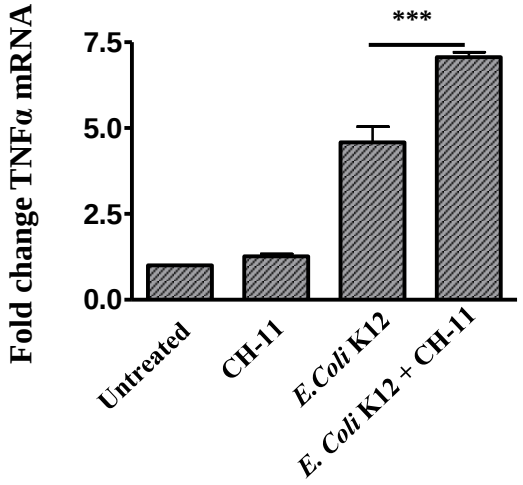


Figure 4.2.1.3 The augmentation of TLR4 and 5-induced IL-8 production by CH-11 also occurs in HT29 and HC116 cells. (a) HT29 cells and **(b)** HCT116 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml Flagellin for 24 hrs. Supernatents were harvested and IL-8 protein concentration determined by ELISA. Statistical analysis was performed and statistical change determined comparing either LPS or flagellin stimulation alone to LPS+CH-11 or Flagellin+CH-11. Values are plotted as Mean ± S.E.M. n=3. ***$p<0.001$, **$p<0.05$; one way ANOVA, Tukeys post hoc t-test.

a)



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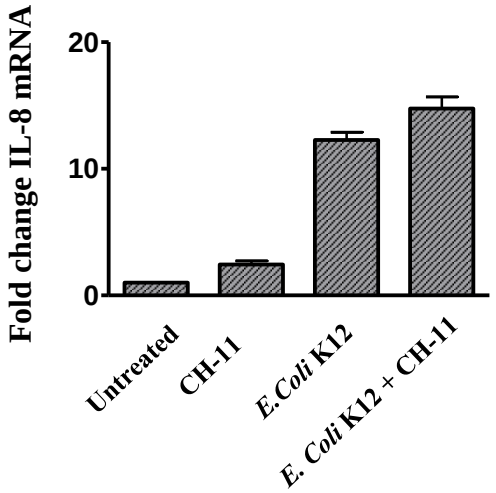


Figure 4.2.2.1 Stimulation of SW480 cells with CH-11 followed by infection by *E.coli* K12 results in augmented cytokine production. (a&b) SW480 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with *E.coli* K12 10:1 (bacteria:cells) for 12 hrs and changes in TNFα and IL-8 were detected by qRT-PCR. Values are plotted as Mean ± S.E.M. n=3. ***p<0.001, as compared to *E. coli* infection only; one way ANOVA, Tukeys post hoc t-test.

4.2.3 The augmented cytokine production observed following stimulation of intestinal cancer cells with CH-11 and TLR4 or TLR5 ligands is independent of cell death.

The Fas/FasL system is best characterised in terms of its role in apoptosis, with some reports showing that cytokine expression occurs concomitantly with apoptotic cell death (Joshi, Kalvakolanu et al. 2003). Moreover, TLRs have been shown to induce apoptosis in certain cell types; for instance TLR5 stimulation can induce apoptosis in intestinal cancer cells (Zheng, Ouaaz et al. 2001). In order to determine if the augmented cytokine production observed occurred concomitantly with, or was independent of, Fas-mediated apoptosis, cells were stimulated with CH-11 and/or LPS or Flagellin, and cell viability and proliferation assessed.

Treatment of SW480 cells with LPS, Flagellin or CH-11 alone, or in combination, did not alter cell viability or cell proliferation relative to untreated cells (Figure 5 a and b). In contrast, cells treated with staurosporine, a natural product isolated from *Streptomyces staurosporeus* that is known to induce apoptosis, resulted in substantially reduced cell viability (reduced by 75%) and proliferation (reduced by 50%) relative to untreated cells (Figure 4.2.3.1 a and b). Since caspase activation is essential for Fas-mediated apoptosis induction, caspase 3/7 activity was also assessed. Caspase 3/7 activity was not increased upon LPS, Flagellin or CH-11 treatment either alone or in combination (Figure 4.2.3.1c), whilst a four-fold induction in caspase 3/7 activity with staurosporine treatment was observed in these cells. In contrast, Jurkat T cells, known to be sensitive to Fas-mediated apoptosis, demonstrated a 6 fold increase in caspase 3/7 activity following CH-11 stimulation relative to untreated control (Figure 4.2.3.1d), indicating that CH-11 is able to induce caspase 3/7 activity in sensitive cells.

In order to confirm that these results were not cell line specific, cell proliferation and caspase 3/7 activity was also measured in HT29 cells following treatment with LPS, Flagellin or CH-11 either alone or in combination. As observed in the SW480 cell line, cell proliferation was not affected by any of the treatments in HT29 cells (Figure 4.2.3.2a), whilst staurosporine treatment reduced proliferation by 90% relative to untreated cells. Similar to SW480 cells, HT29 cells did not exhibit increased caspase 3/7 activity in response to treatment with CH-11, LPS or Flagellin,

whereas staurosporine induced a 3 fold increase in caspase 3/7 activity (Figure 4.2.3.2b).

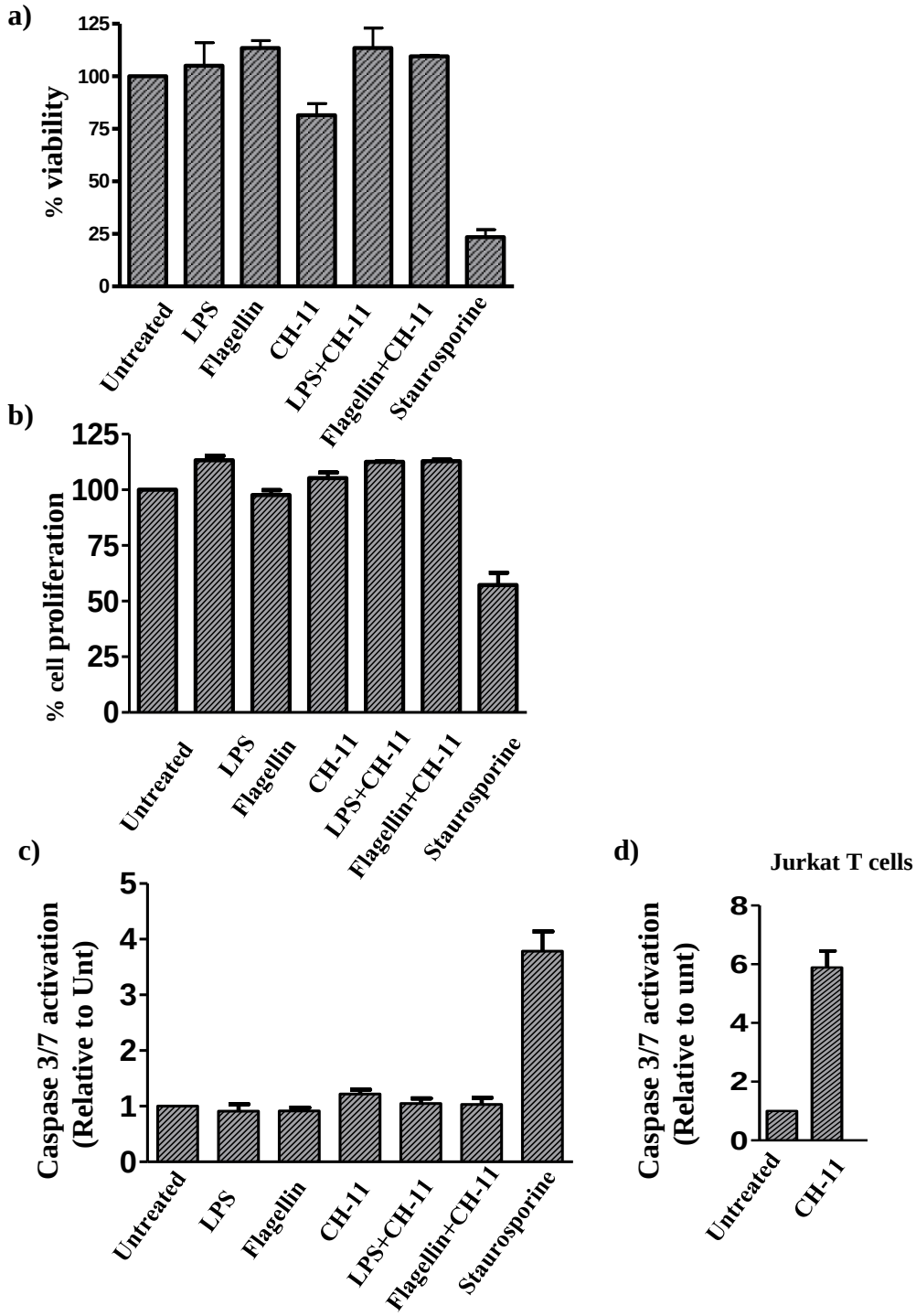
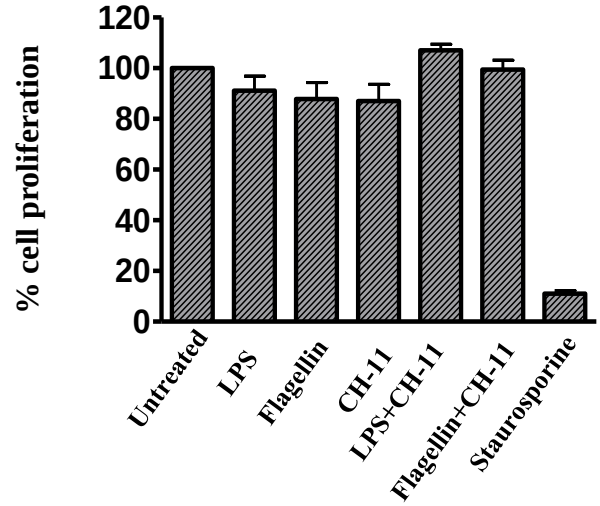


Figure 4.2.3.1 Treatment of SW480 cells with CH-11, TLR4 and/or TLR5 ligands does not alter cell viability, proliferation or caspase 3/7 activation. (a) SW480 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml Flagellin for 8 hrs. Staurosporine treatment was added as a positive control. Trypan blue exclusion was used to determine viability. **(b)** Resazurin reduction was used to measure cell proliferation and **(c)** caspase activation was measured fluorescently. **(d)** Jurkat T cells were treated with 200ng/ml CH-11 as a positive control for caspase 3/7 activation in response to Fas activation.

a)



b)

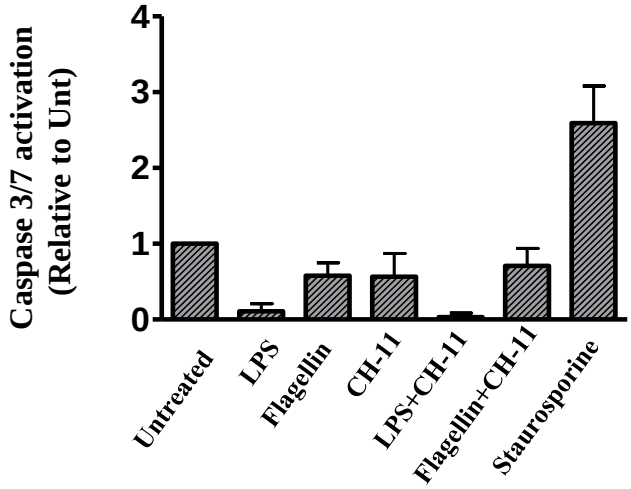


Figure 4.2.3.2 Treatment of HT29 cells with CH-11, TLR 4 and/or TLR5 ligands does not alter proliferation or caspase 3/7 activation. (a) HT29 cells were treated with 200ng/ml agonistic Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml Flagellin for 8 hrs. Staurosporine treatment was included as a positive control. Resazurin reduction was used to measure cell proliferation and **(b)** caspase 3/7 activation was measured fluorescently.

4.2.4 Stimulation of intestinal cancer cells with agonistic anti-Fas reduces the expression of TLR inhibitory proteins.

TLR signalling is regulated by an extensive array of TLR inhibitory proteins. In order to investigate the mechanism by which TLR4 and TLR5-induced cytokine production is augmented by Fas activation, the expression levels of a panel of TLR inhibitory proteins was assessed in SW480 cells following LPS or Flagellin treatment either alone or when pre-treated with CH-11.

Stimulation of SW480 cells with CH-11 reduced the transcription of both TOLLIP and SIGIRR ($p<0.05$) as compared to untreated cells (Figure 4.2.4.1a). This reduced expression was maintained when cells were subsequently treated with LPS or Flagellin. In contrast, the expression levels of two other TLR inhibitors, PPARγ and IRAKM, were unaffected by CH-11 stimulation in SW480 cells (Figure 4.2.4.1b). The change in protein levels of SIGIRR but not IRAKm was confirmed by Western blotting (Figure 4.2.4.1c and d). These findings suggest that the augmentation in TLR4- and TLR5- induced cytokine production by Fas may be due to its ability to downregulate expression of key TLR inhibitory proteins.

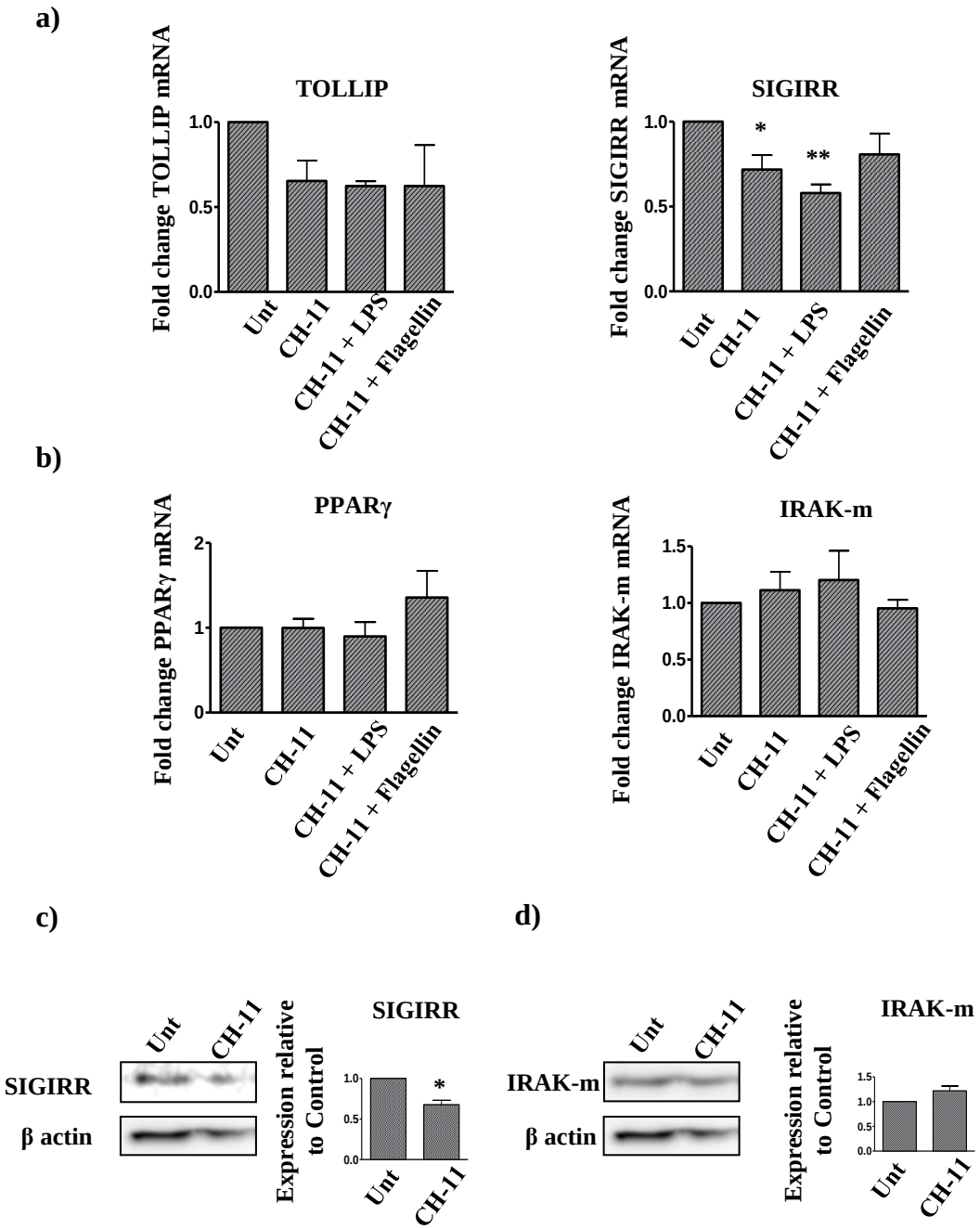


Figure 4.2.4.1 Fas activation reduces the expression of key TLR inhibitory proteins in IECs. (**a and b**) SW480 cells were treated with 200ng/ml agonistic anti-Fas antibody (CH-11) for 1hr followed by stimulation with 100ng/ml LPS or 100ng/ml Flagellin for 4 hrs. Changes in inhibitory proteins were detected by qRT-PCR. (**c and d)** Cells were treated with CH-11 as above for 24 hrs and changes in protein expression were detected by Western blotting and quantified by densitometry. Results shown are representative of three independent experiments. Statistical analysis was performed using one-way Anova, Tukeys post hoc t-test **(a and b)** and paired students *t* test **(c and d)** and statistical change determined compared to untreated control. Values are shown as Mean ± SEM, n=3. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$.

4.2.5 FADD associates with MyD88 in intestinal cancer cells.

Previous studies in macrophages have shown that, in the absence of Fas signalling, the Fas adaptor protein, FADD, is present in the cytoplasm bound to the TLR adaptor protein, MyD88, and this interaction suppresses MyD88-dependant cytokine production [104, 223]. Engagement of Fas was shown to prevent this interaction, releasing MyD88 and thereby promoting TLR-mediated inflammation. Whilst I have shown that downregulation of TLR inhibitory proteins is one potential mechanism whereby stimulation of Fas augments TLR4- and TLR5- induced TNFα and IL-8 production in intestinal cancer cells, I also examined whether FADD and MyD88 interacted in intestinal cancer cells as this may be an additional mechanism underlying the augmentation in cytokine production. Plasmids encoding Flag-tagged FADD and HA-tagged MyD88 (Flag-FADD and MyD88-HA respectively) were over-expressed in HCT116 cells. Expression was confirmed by Western blotting (Figure 4.2.5.1 a and b). Next, the co-immunoprecipitation technique was optimised. HCT116 cells were transfected with either Flag-tagged FADD or HA-tagged MyD88 or co-transfected with both constructs. Following immunoprecipitation with either anti-FLAG or anti-HA, I was able to confirm immunoprecipitation of Flag-FADD (Figure 4.2.5.1c) and MyD88-HA (Figure 4.2.5.1d) by Western blotting respectively. Upon co-transfection with Flag-tagged FADD or HA-tagged MyD88, HA-tagged MYD88 was seen to co-immunoprecipitate with Flag-tagged FADD (Figure 4.2.5.2), thus indicating an association between the two adaptor proteins in intestinal cancer cells.

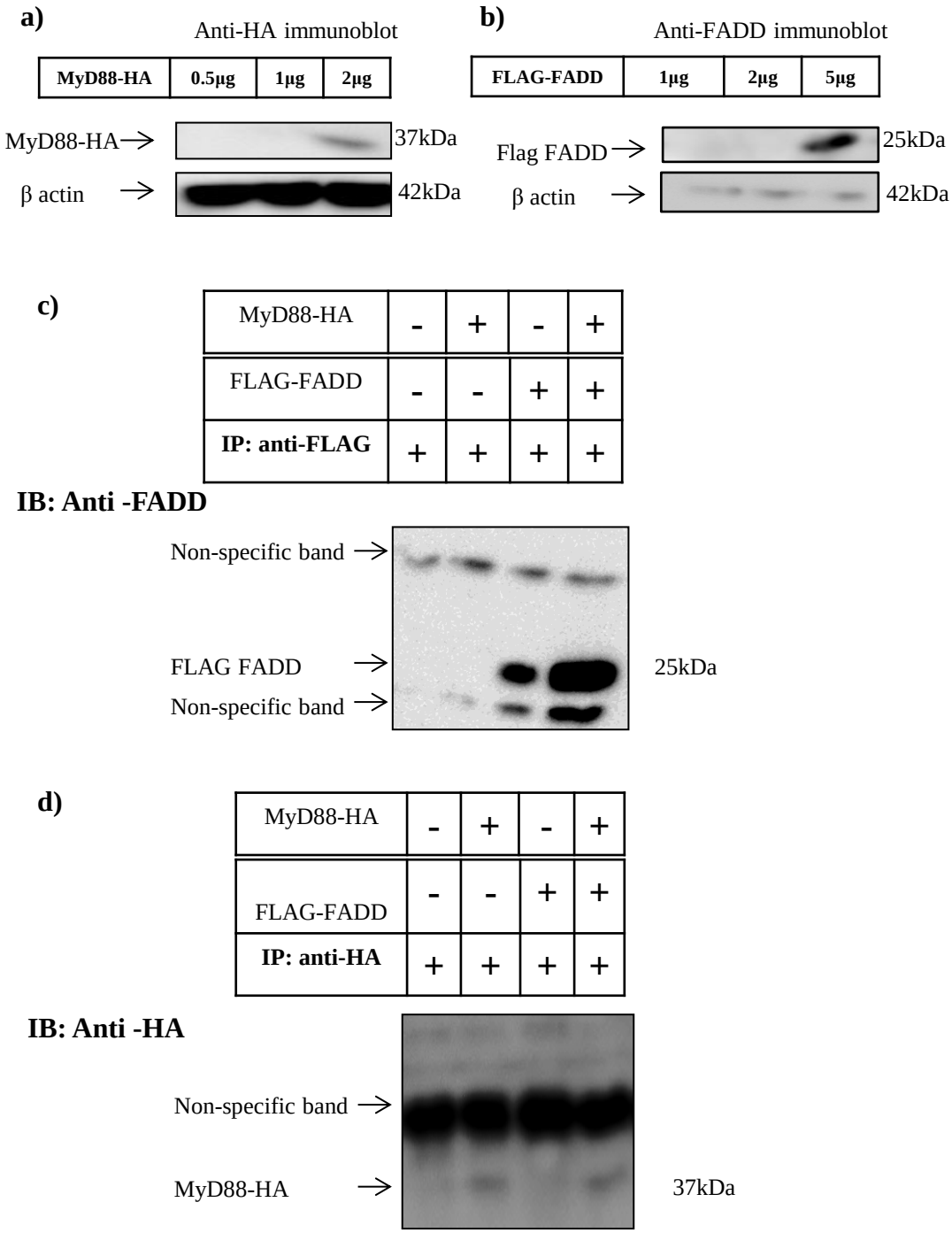


Figure 4.2.5.1 Optimisation of coimmunoprecipitation experiment in HCT116 cells. (a) HCT116 cells were transfected with either 0.5-2µg HA-tagged MyD88 or **(b)** 1-5µg FLAG-tagged FADD constructs. Cell lysates were separated by SDS-PAGE and probed with anti-HA, anti-FADD or anti- β actin specific antibodies as indicated. **(c & d)** Cells were transfected with 2µg MyD88-HA, 5µg FLAG-FADD or co-transfected with both constructs. Immunoprecipitation (IP) was performed with anti-FLAG or anti-HA antibodies. The immunoprecipitates were subjected to immunobloting (IB) with anti-HA or anti-FADD specific antibodies as indicated.

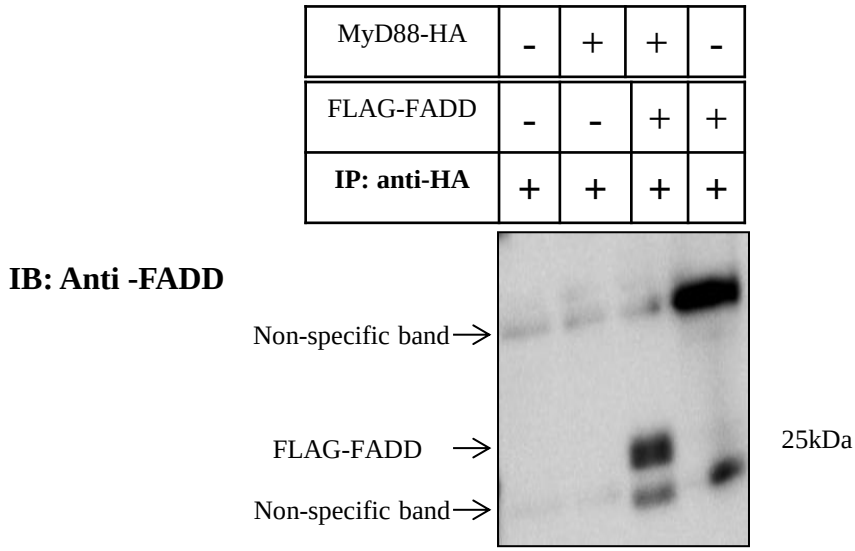


Figure 4.5.5.2 FADD associates with MyD88 in intestinal epithelial cells. Cells were transfected with 2μg MyD88-HA, 5μg FLAG-FADD or co-transfected with both constructs. Immunoprecipitation (IP) was performed with anti-HA antibody. The immunoprecipitate was subjected to immunobloting (IB) with an anti-FADD specific antibody.

4.2.6 Suppression of Fas expression limits the ability of HT29 cells to respond to CH-11

In order to further investigate the potential crosstalk between Fas and TLR4 and 5 in intestinal cancer cells, stable cell lines with reduced Fas expression were generated using lentiviral particles designed to target the human Fas gene, $HT29^{Fas\ shRNA}$ cells. In parallel, HT29 cells were lentivirally transfected with plasmids with a scrambled sequence showing no homology to any known mammalian gene, $HT29^{scr\ shRNA}$ cells. As can be seen in Figure 4.2.6.1a, two distinct HT29 clones transfected with shRNA against Fas, (hereafter referred to as $HT29^{Fas\ shRNA}$ cells #1 and #2) were identified, showing a reduction in Fas expression ($p<0.001$) relative to $HT29^{scr\ shRNA}$ cells by Western blot. To further characterise knock down of Fas expression, a functional assay was performed. CH-11 treatment led to a 3 fold increase in IL-8 production in $HT29^{scr\ shRNA}$ cells (Figure 4.2.6.1b). In contrast, neither $HT29^{Fas\ shRNA\#1}$ or $HT29^{Fas\ shRNA\#2}$ cells were able to upregulate IL-8 production upon CH-11 stimulation ($p<0.001$) (Figure 4.2.6.1c).

Fas is a member of the death receptor superfamily, of which TRAIL and TNF are other members. In order to assess the relative specificity of knock down, the expression levels of these receptors was examined by Western blotting. As can be seen in Figure 4.2.6.1d, the expression of TRAIL R-1 and TNFR-1 is unaltered in $HT29^{Fas\ shRNA\#1}$ cells relative to $HT29^{scr\ shRNA}$ cells.

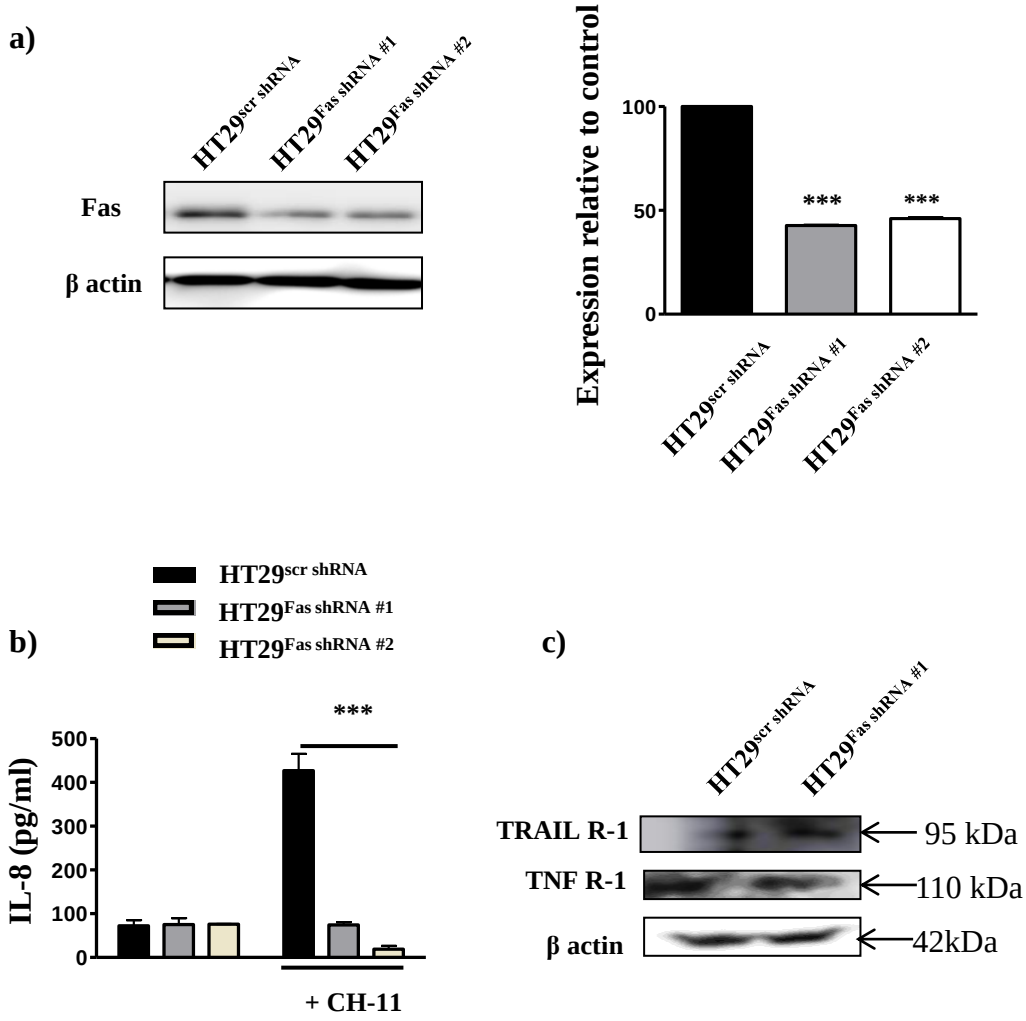


Figure 4.2.6.1 Fas expression was reduced in HT29 cells using lentiviral particles expressing short hairpin RNA targeted against Fas. (a) HT29 cells were transfected with Fas or scrambled control shRNA lentiviral particles in the presence of polybrene. Following selection, stable clones were generated and knockdown of Fas expression was determined by Western blotting and quantified by densitometry. **(b)** Changes in IL-8 production was assessed by ELISA.

(c) Total cellular lysates from HT29 cells were separated by SDS-PAGE, and probed with the specified antibodies. Values are plotted as Mean ± S.E.M. n=3. ***$p<0.001$, as compared to $HT29^{scr\ shRNA}$; one way ANOVA, Tukeys post hoc t-test.

4.2.7 Suppression of Fas expression limits the ability of intestinal cancer cells to respond to TLR4 & TLR5 ligands.

In order to characterise the effect of reducing Fas expression on the response of intestinal cancer cells to TLR ligands, $HT29^{scr\ shRNA}$, $HT29^{Fas\ shRNA\#1}$ and $HT29^{Fas\ shRNA\#2}$ cells were stimulated with LPS and Flagellin. In contrast to the 3 fold induction of IL-8 protein production seen upon LPS stimulation of $HT29^{scr\ shRNA}$ cells, neither $HT29^{Fas\ shRNA\ \#1}$ nor $HT29^{Fas\ shRNA\ \#2}$ cells were able to increase expression of IL-8 in response to LPS ($p<0.001$) (Figure 4.2.7.1). Furthermore, whilst $HT29^{scr\ shRNA}$ cells upregulated IL-8 production in response to Flagellin, neither $HT29^{Fas\ shRNA\ \#1}$ nor $HT29^{Fas\ shRNA\ \#2}$ cells were able to increase expression of IL-8 in response to Flagellin ($p<0.001$).

To determine whether IL-8 secretion in response to other TLR ligands was also affected by Fas suppression, cells were stimulated with the TLR2 ligand, Pam3CSK4, and the TLR9 ligand, CpG DNA. $HT29^{scr\ shRNA}$ cells secreted IL-8 in response to stimulation with CpG DNA, and this response was unaffected by a reduction in Fas expression (Figure 4.2.7.1a). HT29 cells were unable to secrete IL-8 in response to Pam3CSK4 irrespective of Fas expression levels. In contrast, both $HT29^{Fas\ shRNA\#1}$ and $HT29^{Fas\ shRNA\ \#2}$ cells were able to produce IL-8 to a level comparable to that secreted by $HT29^{scr\ shRNA}$ cells in response to TNFα, a known inducer of IL-8.

These results were confirmed in CT26 murine intestinal cancer cells. Fas was stably suppressed in these cells by shRNA ($CT26^{Fas\ shRNA}$) (Figure 4.2.7.1b). As murine cells do not produce IL-8, the induction of CXCL-1, a murine IL-8 homolog, was determined. Consistent with suppression of Fas expression, the $CT26^{Fas\ shRNA}$ cells failed to upregulate CXCL-1 in response to Jo-2 (a murine agonistic anti-Fas Ab) (Figure 4.2.7.1c). The ability of LPS to induce CXCL-1 in $CT26^{Fas\ shRNA}$ was also reduced to basal levels consistent with the findings in HT29 cells. CT26 cells do not express TLR5 [294] and therefore I was unable to assess their response to Flagellin.

In order to confirm that the abrogation in the ability of $HT29^{Fas\ shRNA\#1}$ and $HT29^{Fas\ shRNA\ \#2}$ cells to respond to LPS and Flagellin was not due to any reduction in expression of other proteins following shRNA transfection, the expression levels of

TLR4, 5, 2 and 9 were assessed by Western blotting. The expression level of these receptors was unchanged between $HT29^{scr\ shRNA,}$ $HT29^{Fas\ shRNA\#1}$ and $HT29^{Fas\ shRNA\ \#2}$ cells (Figure 4.2.7.2).

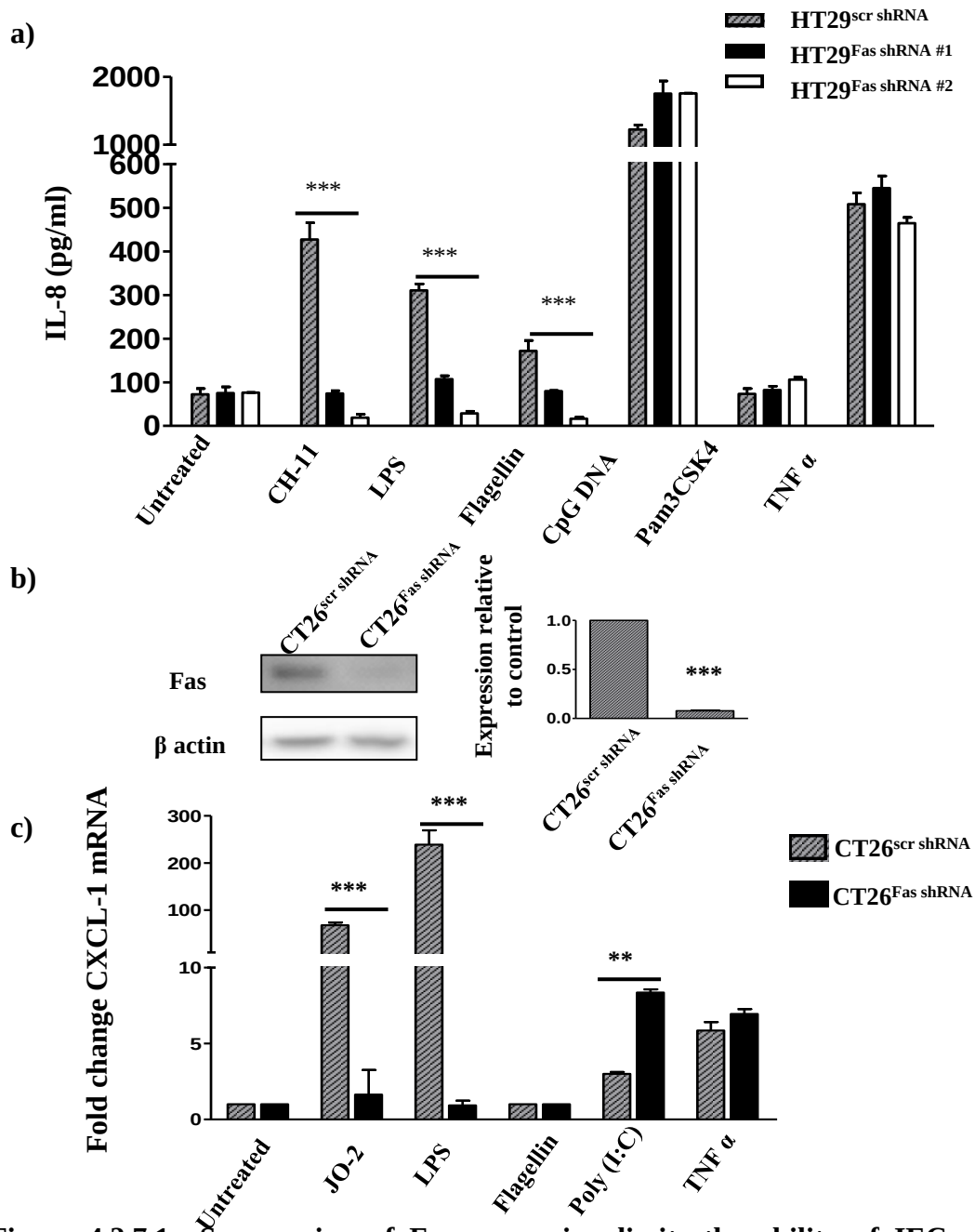


Figure 4.2.7.1 Suppression of Fas expression limits the ability of IECs to respond to TLR4 and TLR5 ligands. (a) HT29$^{scr\ shRNA}$ and HT29$^{Fas\ shRNA}$ clones were treated with either 200ng/ml agonistic anti-Fas antibody (CH-11), 100ng/ml Flagellin, 100ng/ml LPS, 5μM CpG, 10μM Pam3CSK4, or 100ng/ml TNFα for 24 hrs. Cell supernatants were collected and IL-8 protein production determined by ELISA. **(b)** CT26 cells were transfected with Fas shRNA lentiviral particles and stable clones generated. Knockdown of Fas expression was determined by Western blotting and quantified by densitometry. **(c)** CT26$^{scr\ shRNA}$ and CT26$^{Fas\ shRNA}$ clones were treated with either 50ng/ml agonistic Fas antibody (JO-2), 100ng/ml flagellin, 100ng/ml LPS, 1mg/ml Poly I:C or 100ng/ml TNF α for 4 hrs. Changes in CXCL-1 mRNA were detected by qRT-PCR. Values are plotted as Mean ± S.E.M. n=3. ***p<0.001, **p<0.01 as compared to HT29/CT26$^{scr\ shRNA}$; one way ANOVA, Tukeys post hoc t-test.

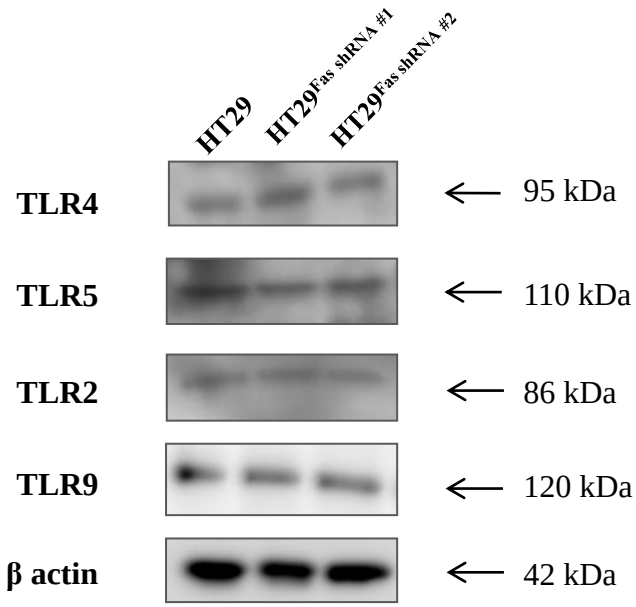


Figure 4.2.7.2 Suppression of Fas expression does not alter expression of TLR2, 4, 5, or 9. Total cellular lysates from HT29 cells were separated by SDS-PAGE, and probed with the specified antibodies. Lysates were probed using β actin as a loading control.

4.2.8 Fas is required for the IL-8 response of HT29 cells to intestinal pathogens *Listeria monocytogenes* (*L. Monocytogenes*) and *Salmonella typhimurium* (*S.typhimurium*).

Given that pre-stimulation with CH-11 followed by *E.coli K12* treatment led to enhanced IL-8 production in SW480 cells, I next investigated whether attenuating Fas expression would alter the response of intestinal cancer cells to intestinal pathogens. Following *S.typhimurium* infection, the production of IL-8 by $HT29^{scr\ shRNA}$ cells doubled relative to uninfected levels (Figure 4.2.8.1). In contrast, both $HT29^{Fas\ shRNA\#1}$ and $HT29^{Fas\ shRNA\#2}$ demonstrated a reduced capability to produce IL-8 in response to *S.typhimurium* infection.

Given that the intestinal cancer cells response to the TLR5 ligand Flagellin was also attenuated upon suppression of Fas expression, the ability of HT29 cells to respond to *L Monocytogenes*, a Flagellin-expressing bacterium was also assessed. $HT29^{scr\ shRNA}$ cells upregulated IL-8 production in response to infection with this bacterium whilst neither $HT29^{Fas\ shRNA\#1}$ nor $HT29^{Fas\ shRNA\#2}$ cells were able to produce IL-8 in response *L. Monocytogenes* infection ($p<0.001$ as compared to $HT29^{scr\ shRNA}$ cells) (Figure 4.2.8.2).

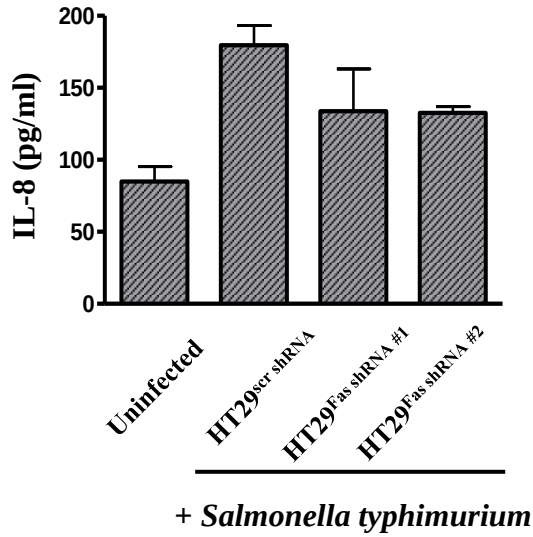


Figure 4.2.8.1 Fas is required for the induction of IL-8 in response to *S. typhimurium* infection in HT29 cells. Confluent HT29 cells were treated with *Salmonella typhimurium* for 3 hrs followed by treatment with gentamycin (50ng/ml). Bacteria were diluted in PBS for infection at multiplicity of infection (MOI) of 10:1. IL-8 protein levels in cell culture supernatants were measured after 24hrs by ELISA. Values are plotted as Mean ± S.E.M. n = 3.

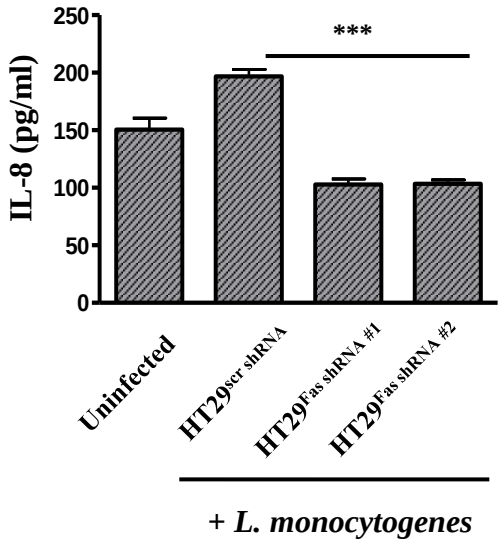


Figure 4.2.8.2 Fas is required for the induction of IL-8 in response to *L. monocytogenes* infection in HT29 cells. Confluent HT29 cells were treated with *Listeria monocytogenes* for 3 hrs followed by treatment with gentamycin (50ng/ml). Bacteria were diluted in PBS for infection at multiplicity of infection (MOI) of 10:1. IL-8 protein levels in cell culture supernatants were measured after 24hrs by ELISA. Values are plotted as Mean ± S.E.M. n=3. ***$p<0.001$, as compared to $HT29^{scr\ shRNA}$; one way ANOVA, Tukeys post hoc t-test.

4.3 Discussion

In this chapter I have demonstrated that extensive cross-talk exists between the Fas and TLR signalling pathways in intestinal cancer cells in terms of cytokine induction in response to bacterial infection. Firstly, co-stimulation with an agonistic Fas antibody, CH-11, in combination with either TLR4 or TLR5 ligands increases expression of TNFα, IL-8 and CXCL-1 over and above the TLR ligand alone. This cytokine production was independent of alterations in viability, proliferation and caspase 3/7 activation suggesting that increased cytokine production occurs independently of Fas-mediated apoptosis. The downregulation in the expression of a number of key negative regulators of TLR signalling by Fas ligation may partly responsible for the altered cytokine production in intestinal cancer cells. Finally, suppression of Fas expression led to a reduction in the ability of intestinal cancer cells to respond to TLR4 and TLR5 ligands, despite these cells still expressing TLR4 and TLR5. Moreover, $HT29^{Fas\ shRNA}$ cells exhibited a reduced ability to express IL-8 upon bacterial infection with either *L. monocytogenes* or *S. typhimurium* suggesting a possible physiological consequence for cross talk between Fas and TLR signalling in intestinal cancer cells.

Allograft studies provided some of the first evidence that the FasL/Fas signalling pathway could trigger inflammation (Kang, Schneider et al. 1997; Turvey, Gonzalez-Nicolini et al. 2000). Ectopic over-expression of FasL in pancreatic and liver cells, resulted in extensive neutrophil recruitment and ultimately, graft rejection in a number of cases [110, 295]. Consistent with a pro-inflammatory function for Fas, I have shown that stimulation of intestinal cancer cells with CH-11 induced the production of IL-8 and TNFα. There have been many other reports demonstrating that activation of Fas signalling can lead to the expression and release of inflammatory factors *in vitro* and *in vivo*, both in lymphoid [107, 218, 220, 296] and non-lymphoid tissue [108, 219, 297]. Similar to the data presented here, Fas stimulation has been shown to lead to the production of IL-8 in a number of cell types including synoviocytes, bronchiolar epithelial cells, and astrocytes [100-102]. A number of other cytokines and chemokines have also been shown to be induced by Fas ligation including interleukin-6 (IL-6) from astrogliomal cells [103, 104], IL-1β from dendritic cells [105], MCP-1 (monocyte chemoattractant protein-1) from pancreatic cells [106], with macrophages reported to secrete all of these cytokines

and more [104, 107]. In terms of the ability of Fas to induce inflammation in epithelial cells, HT29 intestinal cancer cells have been shown to produce IL-8, IL-6 and MCP-1 following engagement of Fas [221, 293]. The data presented here not only support these findings, demonstrating that intestinal cancer cells respond to the agonistic Fas antibody by secreting TNFα, IL-8 and CXCL-1, but also build on the role of Fas in inflammation by demonstrating that co-stimulation with the TLR4 ligand, LPS, or the TLR5 ligand, Flagellin, leads to an augmentation in production of these pro-inflammatory factors. Although Fas has been shown to modulate TLR4-mediated cytokine production in macrophages [104, 298] and endothelial cells [223], to the best of my knowledge, the data presented here is the first time Fas has been shown to modulate TLR4 signalling in intestinal cancer cells. Furthermore, it is the first time that Fas has been shown to augment TLR5-driven production of inflammatory mediators in any cell type, demonstrating a hitherto unappreciated crosstalk between TLR and Fas signalling in intestinal cancer cells.

Moreover, I have shown that not only does Fas activation augment cytokine synthesis by commercially purchased TLR ligands, but that it also enhances IL-8 secretion by the live bacterium, *E. coli* K12. A recent study has shown that Flagellin from an avirulent bacterial strain such as *E.coli* K12 can induce secretion of IL-8 in intestinal cancer cells to a comparable level as that from the virulent strain, *E. coli* O83:H1 [183]. The data presented here not only confirm that the non-pathogenic, *E.coli* K12 strain is capable of inducing a pro-inflammatory response in SW480 cells, but that this is augmented upon Fas receptor pre-stimulation, thus indicating a physiological role for Fas in the immune response to bacterial infection.

IL-8 and CXCL-1 are potent chemotactic factors important for neutrophil migration. Expression of IL-8 has been shown to be upregulated from intestinal cancer cells in response to bacterial entry [182, 299]. Moreover, in a murine infection model, MyD88-mediated CXCL-1 production was shown to trigger neutrophil recruitment to the colonic lamina propria during infection. Since dissemination of commensal intestinal bacteria to mesenteric lymph nodes is increased in *Clostridium difficile* -infected mice that have undergone neutrophil depletion, neutrophil recruitment is thought to mediate defence by preventing dissemination of bystander bacteria to deeper tissues [300]. The data here, showing that intestinal cancer cells are capable of producing increased levels of IL-8 and CXCL-1 upon Fas and TLR4 and TLR5

ligation, indicate Fas activation may play a role in the recruitment of immune cells, such as neutrophils, upon bacterial infection.

I also observed augmentation in TNFα production in response to both Fas and TLR stimulation at the mRNA level. In the normal healthy colon, a number of roles have been ascribed to TNFα including regulation of epithelial cell proliferation [301] and cell survival [302] [303]. In addition to these cell survival roles, TNFα also modulates mucus secretion from intestinal cancer cells [304, 305] and may also actually induce a secretory phenotype in some colonic cells [306], thus promoting intestinal barrier function. Uncontrolled TNFα synthesis, however, has been implicated in colonic tissue damage and the induction of apoptosis in intestinal cancer cells [307, 308]. TNFα synthesis is therefore tightly regulated in the healthy intestine by a variety of mechanisms such as the production of inhibitory cytokines like IL-10. Studies have shown that commensal bacteria that induce the biosynthesis of TNFα from intestinal cancer cells are also strong stimulators of IL-10 secretion from the underlying peripheral blood mononucleated cells, indicating that a negative feedback loop exists, presumably to limit TNF α-mediated pathology [309]. It remains to be determined whether the augmented levels described here upon Fas and TLR co-stimulation facilitates the protective role of TNFα in the colon or contributes to IBD pathology.

Despite being best known for its ability to induce apoptosis, the augmentation in cytokine production seen in intestinal cancer cells upon Fas ligation did not require activation of the effector caspases, suggesting that it occurred independently of Fas-mediated apoptosis. Moreover, cell viability and cell proliferation were unaffected by any of the inducing ligands. SW480 and HT29 cells have previously been shown to exhibit relative resistance to apoptosis induction [310]. Moreover, it is known that, under homeostatic conditions, intestinal cancer cells do not undergo Fas-mediated apoptosis despite co-expression of Fas and FasL [311]. Proteins that mediate resistance include cellular FLICE-like inhibitory protein (c-FLIP) and inhibitor of apoptosis proteins (IAPs). intestinal cancer cells express high levels of cFLIP, which suppresses Fas-mediated apoptosis though its ability to prevent processing of pro-caspase-8 to its mature active form [312, 313]. Thus, under homeostatic conditions intestinal cancer cells are protected against Fas-mediated apoptosis. Recent studies have suggested high expression levels of these traditional

negative regulators of cell death may actually promote alternative consequences of Fas receptor ligation. For example, depletion of cIAP1 or cIAP2 by RNA interference in intestinal cancer cells has been shown to lead to the attenuation of cytokine and chemokine production in response to a NOD agonist [314]. Furthermore, inhibition of IAPs and treatment with Zvad-fmk, a pan caspase inhibitor, dramatically decreased Fas-induced IL-8 and CXCL-1 expression in Hela cells suggesting that IAPs and caspases are required for optimal Fas-induced pro inflammatory cytokine production [221]. Although cytokine production by Fas was shown in this study to occur concomitantly with apoptosis, apoptosis was *not* required for cytokine production indicating that the production of inflammatory mediators can be uncoupled from Fas-mediated cell death [221]. In fact, this group confirmed what I have shown here, that despite being resistant to Fas-mediated apoptosis, HT29 cells are still able to respond to Fas ligation by the secretion of pro-inflammatory cytokines. It may well be that in intestinal cancer cells, the presence of such anti-apoptotic factors act to promote alternative consequences of Fas receptor ligation such as the expression of IL-8 and TNFα as seen in the data presented here.

TLR signalling is tightly regulated in intestinal cancer cells by TLR inhibitory proteins. I therefore examined whether Fas signalling in intestinal cancer cells altered the expression of a panel of TLR inhibitory proteins known to play an important role in intestinal homeostasis. I have shown here that stimulation of Fas in intestinal cancer cells reduced the expression of TOLLIP and SIGIRR by between 20-40%. Despite the relatively modest reduction, similar levels of suppression have previously been shown to relieve TLR signalling. For example, a 30% reduction in SIGIRR expression has been shown to be sufficient to elicit a 50% increase in TNFα production in a murine model of DSS colitis [315]. TOLLIP expression is high in intestinal cancer cells and is increased upon LPS- or LTA-stimulation in these cells [203]. Furthermore, expression levels of TOLLIP were found to be associated with acquired hyporesponsiveness in intestinal cancer cells [180]. Intestinal cancer cells have also been shown to express high levels of SIGIRR [316], and deficiency in SIGIRR increases susceptibility to colitis induced by DSS [290]. In contrast to the downregulation of SIGIRR and TOLLIP expression in response to CH-11 on intestinal cancer cells, the expression levels of PPARγ and IRAKM remained

unaltered by Fas activation. Although not initially characterised for its TLR-inhibitory properties, PPARγ was subsequently shown to inhibit NF-κB activation and reduces inflammation in animal models of colitis [317]. Moreover, PPARγ expression is partially controlled by TLR4 and luminal bacteria signalling suggesting a homeostatic mechanism to prevent aberrant response to LPS [291]. Similarly, IRAKM is induced upon TLR stimulation. Due to its inactive kinase domain, IRAKM is known to be a negative regulator of TLR signalling in macrophages [318]. In the colon, IRAKM-deficient mice are more susceptible to DSS-induced colitis [319]. However, in spite of the fact that IRAKM co-localises with a susceptibility locus for IBD [320], a small scale study of Crohn's patients failed to show an association between mutant IRAKM and UC [321]. So, although all the inhibitors examined play an important role in limiting intestinal inflammation, Fas does not ubiquitously suppress all of them. The reduced expression of some of these inhibitors by Fas activation in intestinal cancer cells, may however explain the augmentation of TLR4 and TLR5-mediated cytokine production of IL-8 and TNFα.

FADD is the primary adaptor domain for Fas. The results presented here show that FADD interacts with MyD88 in intestinal cancer cells, providing further evidence of crosstalk between the TLR signalling pathway and Fas in intestinal cancer cells. FADD has been previously shown to inhibit TLR-mediated inflammation through its interaction with MyD88 and interleukin -1 receptor –associated kinase 1 (IRAK1) in macrophages [104, 223]. FADD was shown to dissociate from MyD88 upon Fas ligation, thus alleviating the inhibition of TLR signalling, leading to the augmentation of the pro-inflammatory response in macrophages. Given that I have shown that FADD also associates with MyD88 in HCT116 cells under unstimulated conditions, the data presented here suggest a similar mechanism may exist in intestinal cancer cells. Unfortunately, I was unable to examine the consequence of Fas ligation on this interaction in intestinal cancer cells as HCT116 cells appeared to have a limited response to Fas stimulation upon plasmid transfection. Had it been possible, I would have been able to determine whether the association between FADD and MyD88 was disrupted upon Fas ligation in intestinal cancer cells, and also to determine whether this complex was inhibitory to TLR-induced cytokine production in intestinal cancer cells. Although further work is required, the data

presented here suggests that the negative regulation of TLR mediated cytokine production by FADD may be common to both macrophages and intestinal cancer cells.

I also examined the contribution of Fas activation to TLR-mediated cytokine production by knocking down Fas expression in intestinal cancer cells. The near complete abrogation of TLR4 and TLR5-induced IL-8 production suggests that Fas ligation plays an important role in TLR4 and 5 signalling in the colon. This may have important consequences for the conclusions that have been drawn from reports that have investigated Fas deficiency in the colon [147]. In one study, two different strains of mice were used in order to examine the cell autonomous function of Fas in IECs: mice with a death domain mutation in the Fas receptor rendering all cells unable to undergo apoptosis (lpr^{cg}), and mice that were Fas deficient specifically in IECs. lpr^{cg} mice showed no difference in disease severity when compared to wild type (WT) mice. However when reconstituted with bone marrow from WT mice, lpr^{cg} mice experienced hypersensitivity to DSS, suggesting that IECs become more sensitive to DSS only when Fas is mutated specifically in these cells. Moreover, mice lacking expression of Fas specifically in IECs were also more sensitive to DSS colitis compared to their WT counterparts. My findings suggest that IEC-specific deletion of Fas or abrogation of Fas signalling would also lead to a decrease in the ability of these cells to respond to TLR4 and TLR5 ligands. Given that TLR4 and TLR5 activation have been shown to have protective and homeostatic roles in the intestine [322, 323], it could be argued that the hypersensitivity to DSS treatment exhibited by both of these models [147] is actually attributable to a loss of these protective mechanisms, rather than a *de facto* consequence of Fas receptor ablation in IECs.

Interestingly, $HT29^{Fas\ shRNA\ \#1}$ and $HT29^{Fas\ shRNA\ \#2}$ cells showed a similar response to TLR9 ligands as $HT29^{scr\ shRNA}$ cells. This may be explained by the fact that Fas does not ubiquitously suppress all TLR inhibitory proteins. For instance, expression levels of IRAKM remain unaltered upon Fas receptor stimulation in intestinal cancer cells. Studies in macrophages revealed that suppression of IRAKM resulted in augmented CpGDNA (TLR9 agonist)-mediated pro inflammatory cytokine production [318] suggesting that IRAKM is a more potent negative regulator of CpG-mediated as opposed to LPS-mediated cytokine production. This may

therefore account for the similarity of IL-8 response from HT29$^{Fas\ shRNA}$ and HT29$^{scr\ shRNA}$ cells to CpGDNA. Alternatively, it may be that TLR9-mediated cytokine production is not regulated by the FADD-MyD88 complex. TLR9 is an endosomally located PRR meaning that unlike LPS and Flagellin, which interact with their respective receptors at the cell membrane, CpG and TLR9 interact in an endosomal vesicle [186, 324]. The vesicular location of the receptor may mean that TLR9 is less susceptible to the cytosolic FADD-MyD88 complex and therefore less receptive to the FADD-mediated negative regulation of MyD88, thus accounting for the unaltered CpG response seen in HT29$^{Fas\ shRNA}$ cells.

Finally, I have shown that the production of IL-8 by intestinal cancer cells in response to *S.typhimurium* and *L.monocytogenes* requires Fas. *Salmonella* is an important pathogenic Gram-negative bacterium, known to cause a wide variety of diseases ranging from mild diarrhoea to severe systemic infections such as typhoid fever. *S.typhimurium* infection in humans commonly manifests as enterocolitis characterized by diarrhoea and neutrophillic infiltration into the intestinal submucosa [325]. Following oral infection, intestinal cancer cells represent the first barrier to be crossed by this pathogen in order to invade and colonize the intestinal tissues and other organs. Flagellin is known to be both necessary and sufficient for induction of nearly all epithelial pro-inflammatory gene expression induced by *S. typhimurium* [325] and I have shown here that intestinal cancer cells upregulate expression of IL-8 in response to infection with *S.typhimurium*. The augmentation of *Salmonella*-mediated IL-8 response by Fas shown here is likely to promote neutrophil infiltration in response to this pathogen, thus indicating an important role for Fas in host defence against pathogens. Similarly, *Listeria monocytogenes* is a ubiquitous, Gram-positive, soil bacterium and facultative intracellular pathogen, which can cause severe food-borne disease in new-borns, pregnant women or other immunocompromised individuals. Although best characterised for recognition by TLR2 [326], NOD1 [327], and NOD2 [328] and most recently, TLR10 [154], *L. monocytogenes* is also a flagellated bacterium and has been shown to induce NFκB activity through TLR5 which is necessary for host defence [329]. It is possible therefore, that the augmentation of IL-8 in response to *Listeria* by Fas ligation in intestinal cancer cells, represents another mechanism of host defence, improving the likelihood of pathogenic clearance.

In conclusion, I have shown that there is extensive cross-talk between Fas and TLRs 4 and 5 in intestinal cancer cells *in vitro*. The data here further advances the evidence in favour of non-apoptotic functions of Fas, in particular a pro-inflammatory role, in intestinal cancer cells. The alterations in TLR-mediated cytokine production shown in this study, upon manipulation of both the activation and expression of Fas, suggest that Fas is required for optimal pro-inflammatory cytokine production in the case of pathogenic infection in the intestine. This may encourage neutrophil infiltration into the colonic mucosa, thus promoting bacterial clearance from the host. It may also be possible that by upregulating TLR-mediated cytokine production, Fas signalling promotes the unresolving inflammatory phenotype of the colon that characterises intestinal disorders of the intestine such as IBD.

6.1 Final Discussion

Despite being constitutively expressed at the basolateral side of all epithelial cells of the colon, the role of Fas in the gastrointestinal tract is still unclear. Fas has been best characterised as a major regulator of the extrinsic pathway of caspase-dependent apoptosis, yet mice lacking Fas and FasL display no abnormalities in their gut mucosa relative to WT mice, indicating that the Fas/FasL signalling system is unlikely to be involved in the physiological turnover of the gut epithelium. Instead, emerging evidence suggests that Fas signalling may play a role in the host response to pathogens, specifically in pathogen clearance.

Elimination of infected cells via programmed cell death is a defence strategy of multicellular organisms against bacteria, viruses, and parasites in a variety of tissues. By killing the infected cell, host cell apoptosis simultaneously confines and eliminates the infection as well as preventing dissemination to underlying tissue [384]. The importance of Fas-triggered cell death in modulating disease severity has been known for some time. In comparison to wild-type (WT) mice, mice deficient in either Fas or FasL infected with *Pseudomonas aeruginosa* succumb to sepsis with increased splenic colonization with this bacteria [385]. These symptoms are associated with a reduction in lung epithelial cell apoptosis and markedly decreased survival. Similarly, studies using *Mycobacterium tuberculosis* [386], *Listeria monocytogenes* [387] *and Helicobacter pylori* [388] have shown that deficiency of either Fas or FasL leads to decreased Fas-mediated apoptosis of host cells and increased disease severity as compared to WT controls. Moreover, apoptosis has been associated with increased bacterial clearance during infection with *Streptococcus pneumoniae* [389]. Increased Fas and FasL expression in tissues that have an associated microbial flora such as the intestine may 'prime' the region by lowering the threshold for Fas-mediated apoptosis, should infection arise. The upregulation of Fas and FasL upon TLR signalling in intestinal cancer cells seen in the data presented here may therefore be a mechanism to promote Fas-mediated apoptosis of infected epithelial cells of the intestine upon pathogenic infection.

Consistent with a role for Fas-mediated apoptosis as a host defence mechanism, recent studies have shown that certain bacteria target host cell apoptosis. For

example, in the genitourinary tract, *Chlamydia trachomatis* secretes chlamydial proteasome-like activity factor, which targets pro-apoptotic proteins for degradation [390]. *Chlamydia trachomatis* is also able to upregulate the inhibitors of apoptosis proteins, thus preventing the release of cytochrome C from mitochondria, an important step in the intrinsic apoptotic pathway [391]. In this way, the obligate intracellular pathogen ensures the survival of the infected epithelial cell, critical for the establishment of a persistent infection. Moreover, *Yersinia pestis (Y.pestis)*, the causative agent of the pneumonic plague, has been shown to secrete a bacterial plasminogen activator protease called *Pla*, which degrades FasL, thus preventing pulmonary caspase-3/7 activation and host cell apoptosis [392]. In addition to *Pla*, *Y.pestis* produces multiple virulence factors that manipulate host defence strategies, including effector proteins that are injected via a Type III secretion system (T3SS) [393]. This syringe-like apparatus delivers bacterial effector proteins directly into the host cell cytoplasm [126]. For instance the T3SS effector protein, *YopM*, inhibits the activation of caspase-1 to prevent cell death via pyroptosis [394, 395]. In the colon, enteropathogenic *Escherichia coli* (EPEC) has been shown to inject the effector protein, NleB, which is capable of antagonising death receptor signalling by binding to, and modifying the death domain of FADD with an N-acetyl glucosamine residue [125]. This prevents DISC formation, prohibiting caspase activation and thus inhibiting cell death [124]. In addition to its role in Fas-mediated signalling, FADD is also an adaptor protein important in transducing the apoptotic signal via other members of the TNF superfamily, including tumour necrosis factor–related apoptosis-inducing ligand (TRAIL) receptors and death receptors 4 and 5 [396]. Indeed, the GlyNacylation of the death domain of FADD by NleB also blocked TRAIL-induced cell death [125]. This ability to prevent apoptosis of the infected cell thus enhances bacterial colonization, increasing the chances of dissemination to deeper tissues and ultimately, to other hosts [125, 397]. Whether these bacteria also target Fas-mediated inflammation is currently unclear. In the data presented here, Fas ligation led to the secretion of proinflammatory mediators such as TNFα and IL-8, suggesting they are predisposed to alternative consequences of Fas receptor ligation.

Inflammation is one of the best characterised host responses to bacterial pathogenic infection and immune cells act in concert to fight and eradicate the pathogen.

Although several studies have shown the upregulation of a number of proinflammatory cytokines in response to Fas ligation, the role of Fas-mediated inflammation in the response to infection has been largely unappreciated thus far. In contrast, signalling via TNFR-1 has been shown to have a prominent role in the immune response to infection as evidenced by several *in vivo* studies. Infection with *Mycobacterium tuberculosis* (*M. tuberculosis)* results in the TNFR1-mediated secretion of TNFα [398]. This pleotropic cytokine participates in host resistance to infection in a number of ways including the induction of chemokines and subsequent innate immune cell recruitment, as well as participating in the activation of macrophages and T cells [399]. The fundamental role that TNFR-1 signalling plays in the control of bacterial infections is evidenced by the fact that TNFR-1 deficient mice die when orally infected with a low-virulent strain of *Toxoplasma gondii* [400]. Furthermore patients with rheumatoid arthritis receiving anti–TNFα therapy demonstrate an increased susceptibility to *M. tuberculosis* infection [401]. However, recent studies suggest that Fas-mediated inflammation may also play an important role in fighting infection, with Fas-mediated signalling resulting in IL-18/IL-1β production from peritoneal exudate cells in response to infection with *Listeria monocytogenes (L.monocytogenes)* [362]. In support of a role for Fas-mediated inflammation in the response to infection, I have shown that intestinal cancer cells demonstrate reduced IL-8 and TNFα production in response to the intestinal pathogens, *L.monocytogenes* and *Salmonella typhimurium (S.typhimurium),* upon Fas receptor downregulation.

Infection with a mutant strain of *Y. pestis* lacking the effector protein *Pla,* not only prevented apoptosis but also led to the induction of proinflammatory cytokines, relative to infection with a WT strain [392]. This suggests that *Pla*-mediated FasL degradation delays induction of pulmonary inflammation compared to that caused by other Gram-negative bacteria [402]. By limiting cytokine production, *Y. pestis* is thought to avoid early detection by the innate immune system which allows for initial bacterial replication and the establishment of infection in a new host environment. Similarly, in addition to its role in preventing host cell death, NleB was also shown to abolish NFκB activation downstream of TNFR-1 activation *in vitro* [125]. NFκB is a central inflammatory mediator, essential for the induction of a variety of inflammatory cytokines in response to pathogenic infection. By

preventing NFκB activation, pathogens may potentially subvert the host immune response. Furthermore, *SseK1*, present in *S. Typhimurium*, is an NleB homologue which also inhibits TNF α-mediated NFκB signalling [125]. Since NleB requires its O-GlcNAc transferase activity for NFκB activation [403], this suggests that GlyNacylation is a previously unappreciated mechanism that bacterial pathogens utilise to subvert the inflammatory response in the host.

Although best known as an apoptotic inducer, Fas has been shown to induce a number of cellular outcomes including proliferation, migration and inflammation and it is unclear what determine whether a cell responds to Fas ligation with apoptosis or inflammation. In the data presented here, I have shown that FADD and MyD88 exist in a complex which may limit the amount of FADD available to bind to the Fas receptor, potentially inhibiting the transduction of the Fas-mediated apoptotic signal. The authors of one study showed that FADD inhibited MyD88-transduced inflammation [223]. The sequestration of FADD by MyD88 may also predispose the cell to non-apoptotic consequences of Fas ligation, such as inflammation. The ability of adaptor molecules to form a complex may be a regulatory mechanism that potentiates inflammation rather than apoptosis upon either TLR or Fas receptor ligation thus allowing for a vigorous inflammatory response against invading pathogens which may be common to those cells at the front line of host defence such as macrophages and intestinal epithelial cells.

Receptor crosstalk has long been recognized as an important determinant of cellular response. *In vivo*, a variety of different cellular signals are received simultaneously and receptor cross-talk represents a means of fine-tuning these signals so as to mediate an appropriate response. For example, both TLR2 and TLR6 co-operate in detecting bacterial components such as peptidoglycan, but do not have identical specificity [404]. In macrophages, co-expression of CD4-TLR2 and CD4-TLR6 resulted in the activation of TNF-α production, whereas expression of either receptor alone was insufficient to induce production of this proinflammatory mediator [404]. Since TLR2 can also form a functional signalling complex with TLR1, this may mean that different TLR pairs may stimulate different signalling pathways, hence allowing macrophages to tailor responses to individual pathogens by producing

specific patterns of inflammatory mediators. Cells may therefore express a combinatorial repertoire of TLRs in order to discriminate among the large number of PAMPs. Crosstalk may also be used to enhance a response. For instance, LPS-TLR4 signalling in polymorphonuclear leukocytes (PMN) has been shown to upregulate TLR2 expression in endothelial cells in an NFκB dependant manner. This promotes the enhanced activation of endothelial cells in response to bacterial components, LPS and PGN, thus amplifying the transmigration of transendothelial PMN to sites of infection [405]. Receptor crosstalk can also modify the original signal. Instead of inducing apoptosis, Fas ligation has been shown to induce proliferation in quiescent liver cells, with the mitogenic signal shown to be due to the FasL-mediated phosphorylation of the EGFR receptor [406], thus potentially playing a role in liver regeneration following injury.

Crosstalk may also have pathological consequences. At sites of macrophage accumulation, autocrine or paracrine Fas signalling has been shown to promote the TLR-mediated production of cytokines and chemokines *in vitro*, thus potentially contributing to chronic inflammation seen in the rheumatoid arthritis joint [104]. In the lung, Fas activation induces rapid, TLR4/IRAK4-dependent release of the proinflammatory molecule, high mobility group box 1, that contributes to Fas-mediated pro-inflammatory cytokine production by macrophages seen in conditions of chronic inflammation [298]. Furthermore, FADD siRNA treatment greatly reduced the development of septic acute lung injury in a mouse model. Specifically, TNFα and IL-6 production was inhibited which, in combination with the reduced cell death, led to a dramatic improvement in survival in FADD siRNA treated animals relative to untreated animals [407], indicating that enhanced inflammatory responses as a result of Fas and TLR crosstalk may influence the initiation of a pathological state. Thus crosstalk between receptors appears to be an important determinant of ultimate cellular consequence, the characterisation of such necessary for efficacious therapy design.

The importance of Fas-mediated inflammation in host response is evidenced by the fact that ALPS patients who lack a functional Fas receptor exhibit an increased susceptibility to invasive bacterial infection [408]. Low circulating counts of memory B cells were found in ALPS patients along with poor anti–*Streptococcus*

pneumoniae (S. pneumoniae) IgM production following administration of a vaccine against the bacterium [409]. These data suggest that antibody deficiency accounts for at least some of the observed vulnerability of ALPs patients to infection by *S. pneumoniae*. Infection is also one of the leading causes of morbidity and mortality in SLE patients [410]. Affected individuals have lower than normal T cell counts and their macrophages display decreased phagocytic capabilities [411, 412]. Cytokines such as TNF α and IL-8 are required for the priming of the adaptive immune response. Thus, in light of the crosstalk shown here leading to the production of these cytokines, suboptimal cytokine levels in response to bacterial challenge may account, at least in part, for the abnormalities in B and T cells seen in SLE patients. This may contribute to an increased vulnerability to infection. Indeed, a small scale study has suggested that a decreased ability to produce IL-8 by neutrophils in SLE patients may predispose patients to infection [413]. Thus, characterising crosstalk is relevant to understanding tissue in both the healthy and diseased state.

Allogeneic bone marrow transplantation (BMT) is used as a treatment for haematological malignancies whereby elimination of residual tumour cells is achieved by the transplantation of mature allogeneic T cells. However, the transplanted T cells are also responsible for the induction of graft-versus-host disease (GVHD), which leads to significant morbidity and mortality despite routine immune suppression [414]. APG101, a novel recombinant human fusion protein consisting of the extracellular domain of Fas and the Fc domain of an IgG1 antibody [348] has been used successfully to prevent GVHD in murine BMT models. APG101 binds to FasL, blocking its ability to activate Fas. Treatment of mice with APG101 resulted in increased survival rates without interfering with the graft-versus-tumour (GVT) effect [415]. This suggests that APG101 therapy might be useful in preventing GVHD by preserving GVT activity without impairing T-cell function.

Given that cell death manipulation by pathogens can promote virulence, inhibitors of specific bacterial virulence factors such as *Pla* of *Y. pestis* or NleB may confer targeted protection against specific bacterial infection [416]. Preliminary studies have been conducted using TRAIL. Exogenous TRAIL administration during pneumococcal pneumonia restores apoptosis levels, and *S. pneumoniae* colonization

is reduced. Furthermore, survival of the host animal was enhanced, demonstrating the potential of targeting apoptosis in order to bolster host defence [417].

Current cancer therapies such as chemotherapy, γ-irradiation, and immunotherapy or suicide gene therapy exert an antitumour effect primarily by triggering the activation of caspases which ultimately leads to apoptosis of the cancerous cells [418, 419]. Whilst the majority of cancers express a low level of Fas, tumours remain largely resistant to Fas-mediated apoptosis. Instead, this basal level of expression is thought to be sufficient to activate pro-tumorigenic signalling pathways, such as those required for survival, proliferation and migration [420], with a low level of Fas in primary colon tumours predictive of metastatic tumour recurrence [421]. Early studies suggested that the levels of Fas expression on colon carcinoma cells may be the ultimate determinant of susceptibility to Fas-dependant apoptosis, indicating that modulating Fas expression levels may be a way to mediate cell death [422]. Indeed, increased expression of Fas, under conditions which p53 is inactivated by chemotherapeutics such as 5FU, has been shown to contribute to the apoptosis of colorectal cancer cell lines *in vitro* and this therapy has garnered some success in patients *in vivo* [423, 424]. However, pre-clinical studies have shown that systemic activation of Fas in mice using an agonistic Fas antibody caused massive apoptosis of hepatocytes, resulting in fatal hepatitis. This acute liver toxicity was subsequently found to be dependent on the Fc receptors of the antibody with mice lacking FcγRIIB completely resistant to Jo2-mediated lethal hepatotoxicity, suggesting that hepatotoxicity may be limited through the use of non-antibody Fas-activating molecules [425]. Accordingly, recombinant mega FasL, or APO010, has been shown to have anticancer activity *in vitro* and has demonstrated an ability to reduce tumour incidence and size in murine xenograft models of multiple myeloma, non-small cell lung and ovarian cancer [347, 426]. Indeed the efficacy of APG101 treatment in combination with the existing radiotherapy protocol in progressive glioblastoma patients is indicative of the potential therapeutic potential of limiting tumour-expressed Fas activation [427]. There is evidence to suggest that crosstalk between the Fas and TLR signalling pathways also exists in tumour cells of neuronal origin, with the concomitant activation of TLR4 reported to inhibit Fas-mediated proliferation and migration in a glioblastoma model [88]. Given that human glioblastomas are highly resistant to conventional brain tumour therapies [428],

taking advantage of the apparent crosstalk TLR4 has with Fas signalling in these cells may be promising in the development of new cancer therapeutics.

6.2 Future perspectives

In this study, I have demonstrated crosstalk between the Fas signalling pathway and the TLR 4 and 5 signalling pathway. Fas is a member of the TNFR superfamily. Other members include TNF-related apoptosis-inducing ligand (TRAIL)-R1 and TRAILR2, both of which contain DD in their cytoplasmic domains and mediate a signalling pathway similar to that of Fas, utilising the FADD adaptor protein. Moreover, TRAIL also activated both apoptotic and non-apoptotic signalling pathways, including inflammation. Another member of this superfamily that contains a DD is TNFR-1. Ligation of TNFR-1 by TNF recruits the adaptor protein TNF receptor type 1-associated (TRADD). Like FADD, TRADD is a death domain containing adaptor protein. TRADD binds to TNFR-1, recruiting TRAF2, TRAF1 and receptor-interacting protein (RIP), resulting in the production of proinflammatory cytokines and chemokines [429]. Following recruitment to TNFR-1, TRADD can alternatively associate with FADD, thereby initiating the caspase-8 activation pathway, leading to apoptotic cell death. Therefore, like Fas, TNFR-1 can also assemble a signalling complex that is capable of both apoptotic and proinflammatory outcomes. Moreover studies have shown that TRADD can play a crucial role in TRIF-mediated NF-κB activation downstream of TLR3 and TLR4 [364, 365, 430], with direct interactions between TRADD and TLR4 observed [364]. Therefore, it is possible that activation of TNFR-1 may also modulate LPS- and poly I:C-induced cytokine production in a similar manner to that of Fas. Taken together, these studies suggest that DD-containing proteins may represent a mechanism to modify TLR-mediated signalling in addition to the vital role they play in the determination of cell fate. Further studies will be required to investigate the modulatory effects of other members of the TNF family, including TNFR-1, TRAIL -R1 and TRAIL-R2 on cytokine production following TLR activation.

I have also shown that targeting Fas and TLR4 may lead to a reduction in tumorigenesis, suggesting that a FasL antagonist, such as APG101 could be used, in conjunction with a TLR4 antagonist, as a novel cancer therapy regime. However, it is important to fully characterise the effects of suppressing Fas activation alone, as well as in combination with other therapies, on colon tumourigenesis in pre-clinical models. Given the potential immunosuppressive role of Fas in tumour development, it would be important to further characterise the immune infiltrate of the tumours.

Tumour-associated neutrophils (TANs) can exert either pro- or anti-tumorigenic activities [431], with neutrophil infiltration known to be an indicator of poor prognosis in colon cancer. It would therefore be worthwhile to determine the phenotype of the infiltrating neutrophils in my study. Indeed, TGFβ has been shown to induce a population of TANs with a pro-tumour phenotype, with TGFβ blockade resulting in the recruitment and activation of TANs with an anti-tumour phenotype [431], suggesting that one may be able to manipulate the tumour infiltrate for therapeutic purposes. In addition, since Fas ligation may promote a generalised immunosuppressive response in the host, identifying and quantifying the relative level of myeloid-derived suppressor cells between tumours generated in this study would indicate whether this is the case in colon cancer. Additionally, cytotoxic T cells (CTLs) are critical components of the immune response to the presence of neoplasia, and CTL activity has been shown to decline with progressive tumour growth [432, 433]. One could therefore determine if the cytotoxic activity of T cells from tumour-bearing mice is altered upon downregulation of tumour-expressed Fas and therefore assess the contribution that tumour-derived Fas plays in promoting an immunosuppressive environment.

Studies such as these would enhance the findings of this thesis which demonstrated that crosstalk exists between Fas and TLRs 4 and 5 in the intestine, both in terms of expression and in cytokine production, which may have physiological and pathological consequences.

7. References

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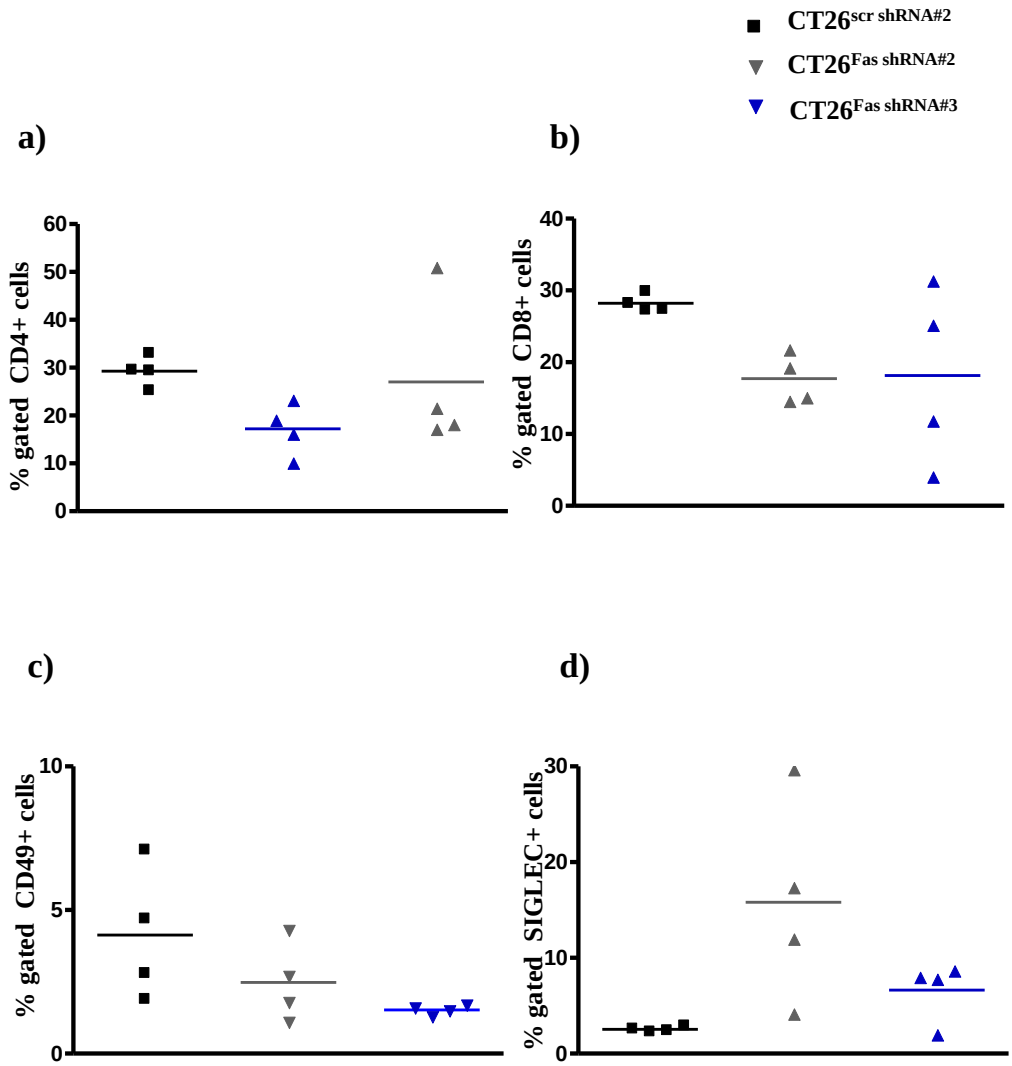
Appendix A



Appendix A. Suppression of tumour expressed Fas does not alter T helper cell, cytotoxic T cell, natural killer cell or eosinophil infiltration into the tumour microenvironment.

Single cell suspensions were isolated from the tumours and surface stained with antibodies against **(a)** CD4, **(b)** CD8, **(c)** CD49b or **(d)** SIGLEC. The percentages of each cell subset were assessed by gating on FSC populations using an Accuri C6 Flow cytometer and analysis was carried out using CFlow software. Values are plotted as Mean ± S.E.M. n=4.

Appendix B

Intestinal Expression of Fas and Fas Ligand Is Upregulated by Bacterial Signaling through TLR4 and TLR5, with Activation of Fas Modulating Intestinal TLR-Mediated Inflammation

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TLRs play an important role in mediating intestinal inflammation and homeostasis. Fas is best studied in terms of its function in apoptosis, but recent studies demonstrate that Fas signaling may mediate additional functions such as inflammation. The role of Fas, and the Fas ligand (FasL), in the intestine is poorly understood. The aim of this study was to evaluate potential cross-talk between TLRs and Fas/FasL system in intestinal epithelial cells (IECs). IECs were stimulated with TLR ligands, and expression of Fas and FasL was investigated. Treatment with TLR4 and TLR5 ligands, but not TLR2 and 9 ligands, increased expression of Fas and FasL in IECs in vitro. Consistent with this finding, expression of intestinal Fas and FasL was reduced in vivo in the epithelium of TLR4 knockout (KO), 5KO, and germ-free mice, but not in TLR2KO mice. Modulating Fas signaling using agonistic anti-Fas augmented TLR4- and TLR5-mediated TNF-α and IL-8 production by IECs. In addition, suppression of Fas in IECs reduced the ability of TLR4 and TLR5 ligands and the intestinal pathogens *Salmonella typhimurium* and *Listeria monocytogenes* to induce the expression of IL-8. In conclusion, this study demonstrates that extensive cross-talk in IECs occurs between the Fas and TLR signaling pathways, with the FasL/Fas system playing a role in TLR-mediated inflammatory responses in the intestine. ***The Journal of Immunology*****, 2014, 193: 6103–6113.**

The intestinal immune system consists of multiple cell types, including intestinal epithelial cells (IECs), which constitute a single monolayer of cells found at the mucosal surface; specialized dendritic cells; and macrophages. In the gastrointestinal tract, IECs are in constant contact with luminal bacteria, their metabolites, and their various inflammatory products. The mucosal surface of the intestinal epithelium has evolved to allow the correct balance of responsiveness, being broadly unresponsive to the presence of the commensal bacteria in the gut lumen while still being able to mount an immune response to the presence of pathogenic bacteria (1). This colonic epithelial cell homeostasis is tightly regulated, as adverse effects can lead to inflammatory conditions such as inflammatory bowel disease or to neoplastic conditions such as colon cancer (2).

TLRs are critical components of the innate immune response that detect microorganisms through the recognition of conserved molecular motifs called pathogen-activated molecular patterns. Expression of TLRs in the intestine has been well characterized. IECs express several TLRs, including TLR2, TLR4, TLR5, and TLR9, with the location of these being restricted to either the apical or the basolateral surface, or both (3). TLR5 is found exclusively on the basolateral surface of IECs, thereby facilitating the detection of its ligand, flagellin (4). Conversely, TLR2, TLR4, and TLR9 have been found at both the apical and the basolateral surfaces (5, 6). Studies performed to characterize signal transduction pathways of TLRs in IECs have shown both inflammatory and homeostatic roles for these receptors following binding of their cognate ligands (7). TLR signaling in the intestine is tightly controlled, as aberrant signaling can give rise to uncontrolled inflammation or impaired defense against pathogens. Indeed, aberrant TLR signaling underlies numerous pathologic conditions of the colon (5).

Several studies have also investigated the role of the death receptor Fas and the Fas ligand (FasL) in IECs (8–10). Fas has been shown to be constitutively expressed in the normal intestinal epithelium, whereas the expression of FasL is more restricted, being most pronounced in the hematopoietic cells. Although best studied in terms of its role in apoptosis, recent studies have identified several nonapoptotic processes for Fas, such as proliferation, migration, invasion and inflammation and tissue regeneration (11). In IECs, ligation of Fas has been shown to induce proinflammatory cytokines in vitro (12), while also having a prosurvival function in dextran sodium sulfite (DSS)–induced colitis (13).

Several studies have shown that a level of cross-talk exists between the Fas and TLR signaling pathways. TLR4 and IL-1R1 signaling is reduced in peritoneal macrophages from Fas-deficient

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The online version of this article contains supplemental material.

Abbreviations used in this article: DSS, dextran sodium sulfite; FADD, Fas-associated protein with death domain; FasL, Fas ligand; FU, fluorescence unit; IEC, intestinal epithelial cell; KO, knockout; PGN, peptidoglycan; shRNA, short hairpin RNA.

(*lpr*) mice, whereas blocking FasL/Fas interactions in macrophages suppresses LPS-induced (TLR4 agonist) and IL-1R1–induced inflammatory cytokine production (14). Furthermore, the Fas adaptor molecule, Fas-associated protein with death domain (FADD), has been shown to negatively regulate TLR signaling (15). Although no study has directly investigated the cross-talk between these pathways in IECs, a recent investigation into Fas signaling in alveolar epithelial cells indicated that Fas-induced inflammation occurred in a MyD88-dependent manner in these cells (16).

In the current study, we sought to determine whether Fas plays a role in the induction of TLR-induced inflammation in IECs. We found that cross-talk exists between these receptors in IECs, in terms of both the expression level of Fas and FasL, and the induction of an inflammatory response. Collectively, these results indicate a new role for Fas signaling in the intestine.

Materials and Methods

Reagents

Agonistic human anti-Fas Ab (CH-11) was obtained from Merck Millipore (Billerica, MA), and Jo-2 (murine) was obtained from BD Biosciences (San Jose, CA). LPS, flagellin, PGN-SA, Pam3CSK4, CpG DNA (ODN 2006), and polyinosinic-polycytidylic acid were purchased from Invivogen (San Diego, CA). Recombinant TNF-α was obtained from PeproTech (Rocky Hill, NJ). The following Abs were obtained from Santa Cruz Biotechnology (Dallas, TX): anti–Fas C-20, Fas X-20, TNFR-1, IGF-IRβ C-20, TLR4 H-80 and TLR5 H-127, and normal rabbit IgG, sc-2027. Anti-FasL Ab (ab15285) was obtained from Abcam (Cambridge, U.K.). Anti–β-actin Ab, TPCA-1, and LY294002 were obtained from Sigma-Aldrich (St Louis, MO). Anti-SIGIRR Ab was obtained from Pro-Sci (Poway, CA), anti–IRAK-m from AbboMax (San Jose, CA), anti-TLR2, and anti-TLR9 Ab from Novus (Littleton, CO)

Cell lines and tissue

HT29, HCT116, SW480, and CT26 colon epithelial cells and Jurkat T cells were obtained from the American Type Culture Collection (Rockville, MD). Cells were maintained in DMEM containing 10% FCS and penicillin–streptomycin. Cells were seeded at 1×10^5 cells per milliliter unless otherwise stated, cultured overnight, and then treated as specified in the figure legends.

Tissue from Swiss Webster wild-type and germ-free mice and from C57BL/6 TLR2, TLR4, and TLR5 knockout (KO) and wild-type mice was kindly provided by the Alimentary Pharmabiotic Centre, University College Cork. Germ-free mice were colonized for 49 d, and tissue was obtained. Tissue from C57BL/6 MyD88 and TRIF KO mice was obtained from Prof. P. Fallon, Trinity College Dublin (Dublin, Ireland).

Generation of Fas$^{low/negative}$ IECs

Cells were transfected with lentiviral particles containing target-specific short hairpin RNAs (shRNAs) against Fas (sc-29311-V) or control lentiviral particles containing scrambled shRNA (sc-108080) (Santa Cruz Biotechnology), according to the manufacturer's instructions. Briefly, cells were seeded in 12-well plates at a concentration of 7.5×10^3 cells per milliliter. Cells were infected 24 h later with lentiviral particles in the presence of 4 μg/ml polybrene, and cultured in selection medium containing 6–8 μg puromycin until resistant clones could be identified. Resistant clones were selected by limiting dilution. Knockdown of Fas expression was determined by Western blotting and functional analysis.

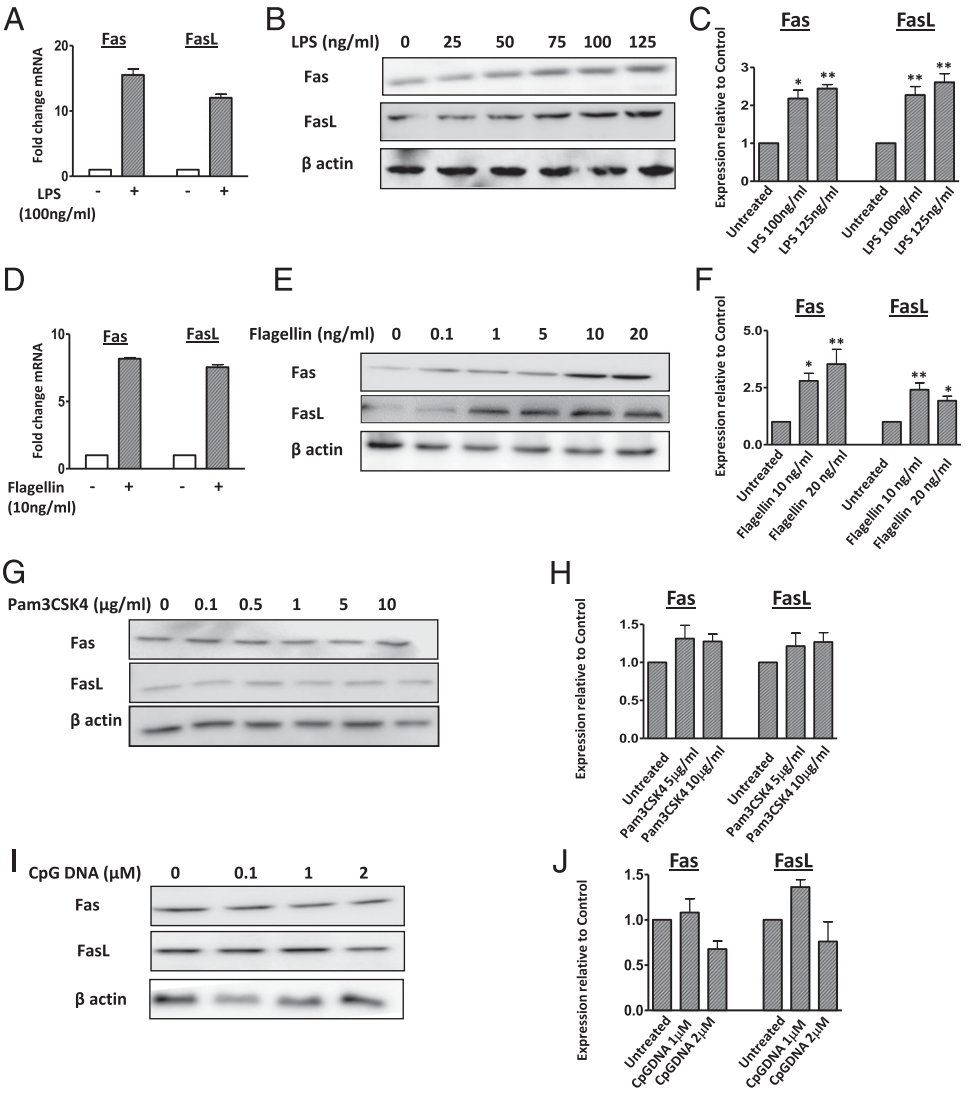

FIGURE 1. Stimulation with LPS and flagellin, but not Pam3CSK4 or CpG DNA, upregulates Fas and FasL expression in SW480 cells. SW480 cells were seeded at a concentration of 1×10^5 cells per milliliter. Cells were treated with increasing concentrations of TLR agonists for 24 h, and Fas, FasL, and β-actin were detected by RT-PCR (**A** and **D**). Changes in protein expression were detected by Western blotting (**B**, **E**, **G**, and **I**), with results shown representative of three separate experiments. Changes in protein expression were quantified by densitometry (**C**, **F**, **H**, and **J**), with analysis performed on four independent experiments. Values are shown as mean ± SEM ($n = 4$). Statistical analysis was performed, and statistical change was determined relative to untreated control. $^*p < 0.05$, $^{**}p < 0.01$.

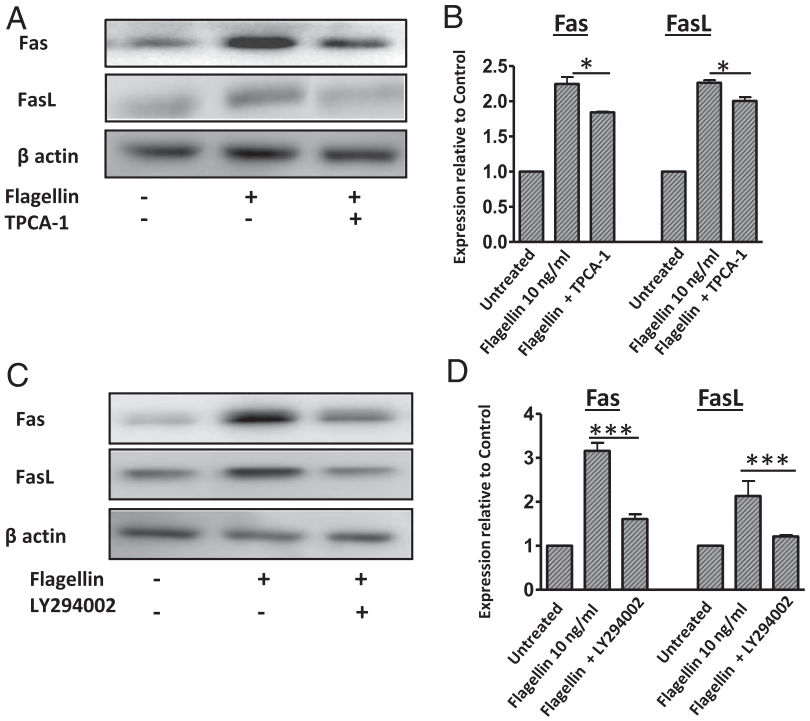

FIGURE 2. Fas and FasL induction by flagellin occurs in an NF-κB– and PI3K-dependent manner. SW480 cells were seeded at a concentration of 1×10^5 cells per milliliter. Cells were pretreated with either 10 μM TPCA1 or 25 μM LY294002, followed by flagellin (100 ng/ml) for 24 h, and Fas, FasL, and β-actin were detected by Western blotting (**A** and **C**), with results shown representative of three separate experiments. Changes in protein expression were quantified by densitometry (**B** and **D**), with analysis performed on three independent experiments. Values are shown as mean ± SEM ($n = 3$). Statistical analysis was performed, and statistical change was determined comparing flagellin stimulation alone with flagellin + TPCA1 or flagellin + LY294002. $*p < 0.05$, $***p < 0.001$.

Bacterial cell culture and infection

The *Listeria monocytogenes* strain EGD (serotype 1/2a) was a gift from Prof. C. Hill (University College Cork, Cork, Ireland). The *Salmonella typhimurium* strain SJW1103 (wild-type) was a gift from Prof. P. O'Toole (University College Cork). *L. monocytogenes* was grown to the logarithmic growth phase in brain–heart infusion broth (Sigma-Aldrich) at 37°C, shaking at 200 rpm, whereas *S. typhimurium* was grown in Luria–Bertani broth (Sigma-Aldrich). Bacteria were diluted in PBS for infection at multiplicity of infection of 10:1. IECs were seeded overnight at 5×10^5 cells per milliliter and cultured with *L. monocytogenes* or *S. typhimurium* for 3 h. Supernatant was removed and replaced with gentamicin-containing media (50 ng/ml), and 24 h later cell culture supernatant was harvested.

The *Escherichia coli* K-12 strain was obtained from the Alimentary Pharmabiotic Centre, University College Cork. IECs were seeded overnight at 5×10^5 cells per milliliter, stimulated for 1 h with CH-11(100 ng/ml), and cultured with *E. coli* K-12 strain (10:1, bacteria/cells) for 8 h. Cells were lysed for subsequent RT-PCR analysis.

Proliferation assay

Cell proliferation was measured by resazurin reduction (17). Cells were seeded at 2×10^5 cells per milliliter in 96-well plates. After incubation for 24 h, medium supplemented with 44 μM resazurin was added, and resazurin reduction to resorufin was measured fluorometrically using a GENios plate reader (Tecan, Grodig, Austria) and Xfluor spreadsheet software. Results obtained were expressed in fluorescence units (FU) and percentage viability was calculated as follows: (FU treated/ FU control) × 100. Values were normalized relative to the untreated cells.

Western blotting

Cells were lysed for 1 h on ice in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 1% Triton X-100, supplemented with complete protease inhibitors (Roche Diagnostics). The protein content of each sample was analyzed using the BCA Protein Assay Kit (Pierce, Rockford, IL). Equal amounts of protein were separated on a 10% SDS–polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were probed overnight at 4°C with primary Ab. As an internal control, all membranes were subsequently stripped of the first Ab and reprobed with anti–β-actin–specific Ab (Sigma-Aldrich). Protein bands were analyzed using ImageJ (National Institutes of Health, Bethesda, MD; http://imagej.nih.gov/ij/, 1997-2012). Changes in protein expression were determined after normalizing the band intensity of each lane to that of β-actin.

RT-PCR

Total cellular RNA was isolated using the GenElute Mammalian Total RNA Mini Kit (Sigma-Aldrich) according to the manufacturer's instructions. cDNA was synthesized using the Bioline kit (London, U.K.). RT-PCR was performed using an Applied Biosystems PRISM 7500 PCR system (Applied Biosystems, Life Technologies, Carlsbad, CA) and TaqMan Gene Expression Master Mix and the following gene expression Taqman primer-probe sets (Applied Biosystems): Fas, Hs00236330_m1; FasL, Hs00181225_m1; IL-8, Hs99999034_ml; TNF-α, Hs00174128_ml; GAPDH, 4352934E. RT-PCR for Tollip, SIGIRR, IRAK-M, PPARγ, 14-3-3 ε, and CXCL-1 was performed using the LightCycler480 System (Roche, West Sussex, U.K.). Individual PCR primer pairs and probes were designed using the Roche Universal ProbeLibrary Assay Design Centre (www.roche-applied-science.com/sis/rtpcr/upl/adc.jsp). Primer sequences and probe combinations are provided in Table I. All samples were run in triplicate, and relative quantitation was calculated using the $2^{-\Delta\Delta Ct}$ method. Transcript levels were normalized to the amount of GAPDH/β-actin mRNA, and expression levels shown as fold induction relative to untreated.

Caspase 3/7 assay

Cells were seeded overnight in black flat-bottom 96-well plates at a density of 20,000 cells per well, treated with CH-11 for 1 h and subsequently with 100 ng/ml flagellin or 100 ng/ml LPS for 24 h, or were treated with each agonist separately. Apo-ONE caspase-3/7 reagent was added, and following 1 h incubation, fluorescence (485 excitation, 530 emission) was measured using a GENios Microplate Reader (Tecan Group, Männedorf, Switzerland). Changes in caspase 3/7 activation were normalized relative to untreated cells.

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissues were deparaffinized in xylene and rehydrated prior to analysis. Ag retrieval was performed by microwave irradiation in 0.01 M citrate buffer, pH 6.0. Slides were washed twice for 5 min in a wash buffer containing 50 mM Tris-Cl, pH 7.6; 50 mM NaCl; 0.001% saponin. Endogenous peroxidase was quenched with 3.0% hydrogen peroxide in methanol for 10 min. Slides were washed as before, except that the wash buffer for this and all subsequent steps included 1% normal goat serum. Nonspecific binding was blocked using 5% normal goat serum in wash buffer for 1 h. Sections were incubated overnight at 4°C with primary Ab or normal rabbit IgG (sc-2027). Ab binding was localized using a biotinylated secondary Ab contained within the VECTASTAIN Elite ABC detection kit (Vector Laboratories, Burlingame, CA). Slides were counterstained with hematoxylin.

A parallel negative control was also performed, using rabbit IgG instead of the primary Ab.

IL-8 ELISA

Supernatants were harvested after 24 h, and IL-8 levels were determined in triplicate by ELISA (eBioscience, San Diego, CA) according to the manufacturer's protocol.

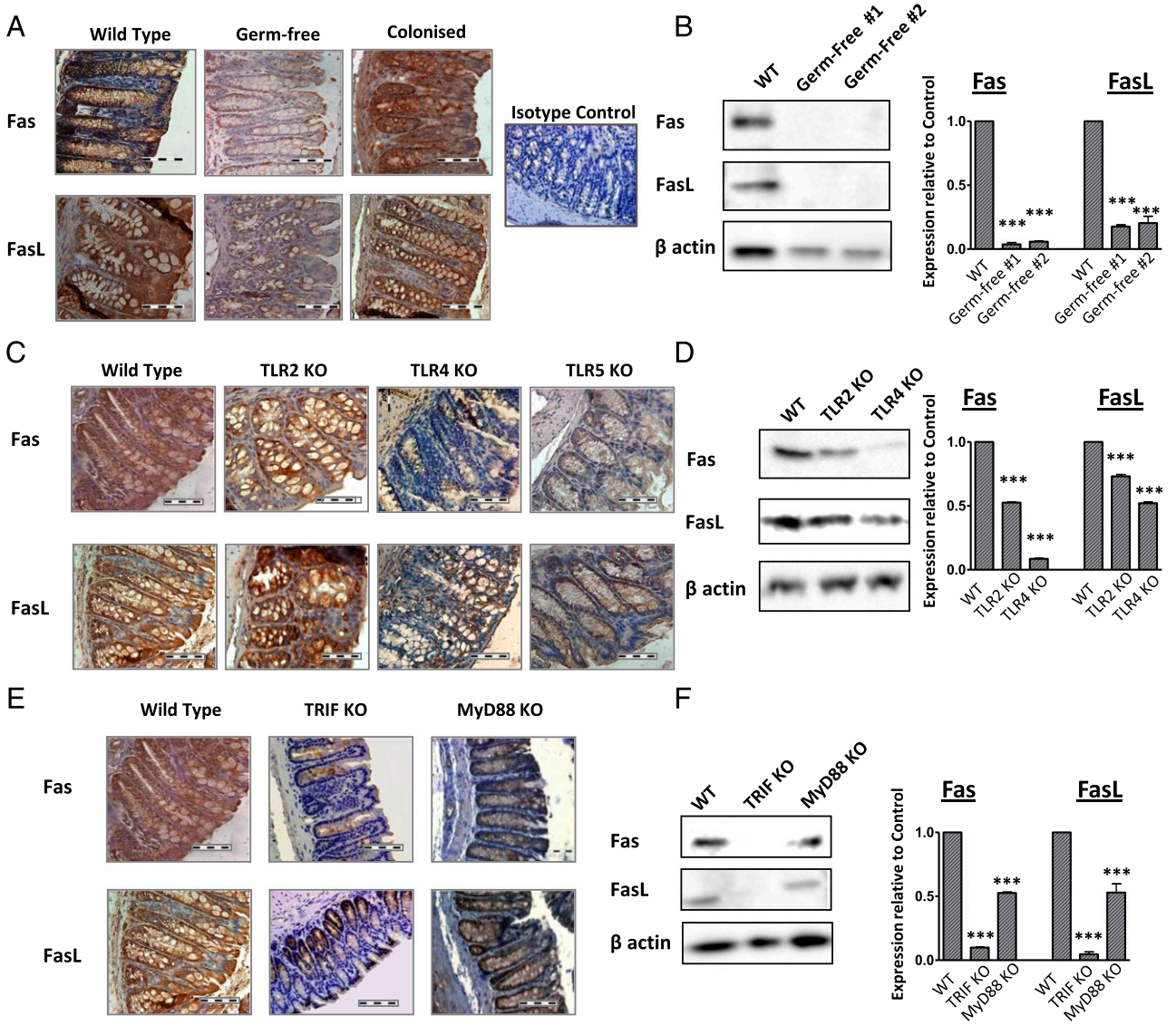

FIGURE 3. Fas and FasL expression is reduced in the distal colons of germ-free, TLR4KO, TLR5KO, TRIFKO, and MyD88KO mice. Immunoperoxidase staining for Fas and FasL was performed on paraffin-embedded colonic tissue sections obtained from wild-type, germ-free, and germ-free mice colonized for 49 d (**A**); wildtype, TLR2KO, TLR4KO, and TLR5KO mice (**C**); and MyD88KO and TRIFKO mice (**E**). Scale bars, 100 μM. Data shown are representative of colonic tissue obtained from five mice per group. Protein was extracted from colonic tissue, and changes in Fas, FasL, and β-actin were detected by Western blotting. (**B**, **D**, and **F**). Data shown are representative of tissue from five mice. Changes in protein expression were quantified by densitometry. Values are shown as mean ± SEM ($n = 5$). ***$p < 0.001$.

Statistics

Experiments were performed a minimum of three times in triplicate. Results were statistically evaluated using one-way Anova with a Tukey posttest, or by the Student paired t test. The p values < 0.001 are indicated by three asterisks (***). The p values < 0.01 are indicated by two asterisks (**). The p values < 0.05 are indicated by one asterisk (*).

Results

Ligands for TLR4 and TLR5, but not TLR2 or TLR9, increase the expression of Fas and FasL in IECs in an NF-κB– and PI3K-dependent manner

Because TLRs are well characterized as key innate immune sensors in the intestine, to investigate cross-talk between Fas and TLR signaling, we first sought to determine whether TLR stimulation upregulated Fas or FasL expression in IECs. SW480 human IECs were selected, as they have been previously shown to express TLRs 1–9 (18). We confirmed protein expression of TLR2, TLR4, TLR5, and TLR9 by Western blotting (Supplemental Fig. 1). Cells were stimulated with the TLR4 ligand, LPS, which upregulated Fas and FasL expression in a dose-dependent manner, at both the mRNA (Fig. 1A) and the protein levels (Fig. 1B, 1C). The TLR5 ligand, flagellin, also upregulated Fas and FasL in a dose-dependent manner in SW480 cells (Fig. 1D–F). In contrast, despite expressing TLR2 and TLR9, neither stimulation with Pam3CSK4, the TLR1/2 ligand (Fig. 1G, 1H), nor transfection with CpG DNA, the TLR9 ligand (Fig. 1I, 1J), altered Fas or FasL expression. We confirmed that SW480s could indeed respond to stimulation with Pam3CSK4 and CpG DNA. Although IL-8 expression did not change in response to stimulation with either ligand, we observed an increase in the gap-junction protein Connexin-43 upon TLR2 ligation and an increase in the Wnt-signaling protein Frizzled5 in response to TLR9 stimulation (Supplemental Fig. 2A). Both these genes have been previously shown to be activated by Pam3CSK4 and CpG DNA, respectively, in IECs (6, 19). We also confirmed the lack of induction using a different TLR2 ligand, peptidoglycan (PGN). Similar to results seen with Pam3CSK4, no induction of Fas was observed following stimulation with PGN (Supplemental Fig. 2B), whereas robust in-

duction of IL-8 was observed following stimulation of SW480 cells with PGN, indicating that these cells are capable of responding to PGN (Supplemental Fig. 2C).

TLR4 and TLR5 activate downstream signaling pathways, such as the NF-κB signaling pathway, the MAPK, and the PI3K/Akt pathway in response to stimulation, and both the FasL and Fas promoter regions contain NF-κB binding sites (20–22). To elucidate the signaling pathway downstream of TLR5-induced expression of Fas and FasL, SW480 cells were pretreated with inhibitors of either the NF-κB or the PI3K pathway prior to stimulation with flagellin. We found that TPCA-1, an IKKβ inhibitor, reduced flagellin-induced upregulation of both Fas and FasL expression ($p < 0.05$) (Fig. 2A, 2B), albeit to a low level. Pretreatment of cells with LY294002, a PI3K inhibitor, also significantly prevented flagellin-induced Fas and FasL expression ($p < 0.001$) (Fig. 2C, 2D).

Fas and FasL expression is reduced in the colons of germ-free, TLR4, and TLR5 KO mice

To determine the in vivo relevance of the induction of Fas and FasL expression by TLR4 and TLR5 ligands, colonic tissue from conventionally reared mice was assessed for Fas and FasL expression. Fas and FasL were found to be expressed in a uniform fashion extending from the base to the surface of the colonic crypts (Fig. 3A). In contrast, expression was reduced in the colonic epithelium of germ-free mice, as assessed by both immunohistochemistry (Fig. 3A) and Western blotting (Fig. 3B), consistent with the lack of exposure of the IECs to commensal flora and thus TLR ligation. Colonization of germ-free mice, however, restored Fas and FasL expression to levels seen in conventionally reared animals, suggesting that Fas and FasL expression is, at least, partially dependent on colonization of the colon by commensal bacteria (Fig. 3A).

As we had observed that stimulation through TLR4 and TLR5 induced the expression of both Fas and FasL in SW480 cells in vitro, we next examined colonic tissue taken from TLR4KO and TLR5KO mice. In contrast to the immunohistochemical staining pattern observed in wild-type mice, colonic tissue from both TLR4KO and TLR5KO mice demonstrated a marked reduction in expression of both Fas and FasL (Fig. 3C). This reduction in expression of Fas and FasL in TLR4KO colonic tissue was confirmed by Western blotting (Fig. 3D). Consistent with our in vitro findings, Fas and FasL expression was unchanged in the epithelial colonic cells of TLR2KO mice, although it appeared reduced in the Western blot (Fig. 3C, 3D). This difference in expression may be due to the mixed population of cells represented in the Western blot compared with the specific IEC immunohistochemical staining. Expression of TNF receptor 1 (another member of the death receptor family) or the insulin growth receptor 1β, however, was unaltered in TLR4KO and TLR5KO mice (Supplemental Fig. 3A).

We subsequently examined whether the observed change in Fas and FasL expression in the colonic tissue of TLR4 and TLR5 KO mice was present in other tissues. The expression pattern in the lung was similar to that observed in the colon; that is, expression of Fas and FasL was downregulated in the lung epithelium (see arrows) of both TLR4KO and TLR5KO mice (Supplemental Fig. 3B). In contrast, expression of Fas and FasL was unchanged in the cerebellum or kidneys (Supplemental Fig. 3C, 3D, respectively). This observation suggests that the regulation of Fas and FasL by TLR4 and TLR5 is tissue specific and may be dependent on the presence of the commensal flora.

These findings indicate that IECs show specificity in their ability to upregulate Fas and FasL expression in response to TLR ligation. One possible explanation for this specificity may be differences in the signaling pathways activated by the TLRs. Although all four TLRs examined in this study (TLR2, TLR4, TLR5, and TLR9) use the TLR adaptor protein MyD88, only TLRs 4 (23) and 5 (24) use the adaptor molecule TRIF. This utilization of TRIF by TLR5 was shown specifically in IECs (24).Therefore we next examined Fas and FasL expression in TRIF and MyD88 KO tissue. Expression of both Fas and FasL protein was significantly reduced in TRIF KO tissue, as assessed by both immunohistochemistry (Fig. 3E) and Western blotting (Fig. 3F). Expression was also reduced, albeit to a much lower level, in MyD88 KO tissue (Fig. 3E, 3F). These findings indicate that signaling through TRIF by TLR4 and TLR5, but not TLR2 and TLR9, agonists may be responsible the induction of Fas and FasL in IECs.

Stimulation of IECs with agonistic anti-Fas Ab and TLR4 or TLR5 ligands results in augmented cytokine production

As TLR and Fas signaling can induce the production of inflammatory cytokines, we subsequently examined whether stimulation of both receptors in IECs resulted in an altered cytokine profile. To investigate this, SW480 cells were treated with the agonistic anti-Fas Ab, CH-11, followed by stimulation with either LPS or flagellin. Cytokine gene expression was initially analyzed by quantitative RT-PCR (Table I). Stimulation with CH-11, LPS, or flagellin alone induced the expression of TNF-α and IL-8, whereas stimulation with a combination of CH-11 and either TLR agonist resulted in augmented transcription of these cytokines (Fig. 4A, 4B). Similar findings were observed upon stimulation of SW480 cells with CH-11 and the Gram-negative bacterium *E.coli*, strain K-12, which would be expected to stimulate cells through TLR4 (Fig. 4C, 4D).

The augmented production of IL-8 in SW480 cells was confirmed at the protein level by ELISA, with stimulation of the cells with both CH-11 and LPS or flagellin resulting in a statistically significant increase in IL-8, relative to either treatment alone (Fig. 4E). We also confirmed that the augmented cytokine production observed upon Fas and TLR ligation was not specific to SW480 cells. HT29 and HCT116 IECs, which express TLR4 and TLR5 (Supplemental Fig. 1), were treated in the same fashion as the SW480 cells. IL-8 protein secretion was significantly augmented following stimulation of HT29 and HCT116 cells with flagellin and CH-11

Table I. qRT-PCR primers and corresponding UPL probe numbers

Gene	DNA Sequence (Sense 5′–3′)	DNA Sequence (Antisense 5′–3′)	UPL Probe No.
β-actin	ATTGGCAATGAGCGGTTC	TGAAGGTAGTTTCGTGGATGC	11
14-3-3 ε	TCTGGTGTACCAGGCGAAG	CCATCCCTGCTACTTTCTTCA	22
SIGIRR	AGCTCTTGGATCAGTCTGCTG	GGCCCTATCACAGACACCTG	36
Tollip	CAACCTCGTCATGTCCTACG	GCTGGTACACTGTTGGCATC	38
IRAK-m	AGAGCTCTGCGCTGTTCTG	GCTGCTTGAAAGTCTCTCTCTGC	26
PPARγ	TGACAGGAAAGACAACAGACAAAT	GGGTGATGTGTTTGAACTTGATT	7
Murine CXCL-1	GACTCCAGCCACACTCCAAC	TGACAGCGCAGCTCATTG	83
Murine β-actin	AAGGCCAACCGTGAAAAGAT	GTGGTACGACCAGAGGCATAC	56

UPL, Universal ProbeLibrary.

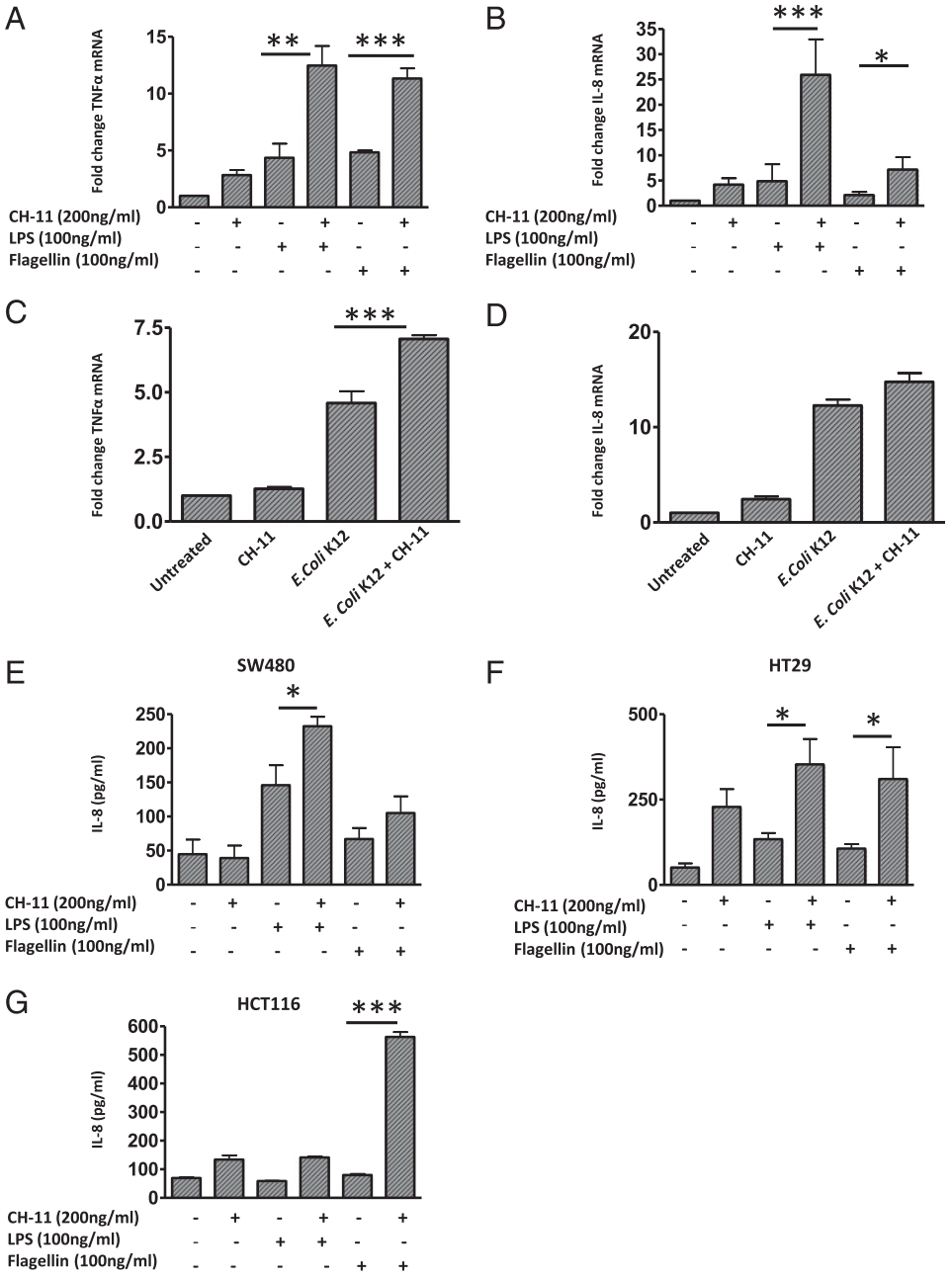

FIGURE 4. Stimulation of SW480, HT29, and HCT116 cells with agonistic anti-Fas Ab and TLR agonists augments cytokine production. Cells were treated with 200 ng/ml agonistic anti-Fas Ab (CH-11) for 1 h followed by stimulation with 100 ng/ml LPS or 100 ng/ml flagellin for 8 h. Changes in TNF-α (**A**) and IL-8 (**B**) were detected by qRT-PCR. Cells were treated with 200 ng/ml agonistic anti-Fas Ab (CH-11) for 1 h, followed by stimulation with *E. coli* K-12 10:1 (bacteria/cells) for 12 h, and changes in TNF-α (**C**) and IL-8 (**D**) were detected by qRT-PCR. SW480 (**E**), HT29 (**F**), and HCT116 (**G**) were treated with CH-11, as above, followed by stimulation with LPS or flagellin for 24 h. Cell culture supernatants were harvested, and changes in IL-8 secretion were detected by ELISA. Statistical analysis was performed, and statistical change was determined comparing either LPS or flagellin stimulation alone with LPS + CH-11 or flagellin + CH-11. Values are shown as mean ± SEM ($n = 3$). $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(Fig. 4F, 4G, respectively), and with LPS and CH-11 in HT29s (Fig. 4F), compared with either stimulation alone.

The augmented cytokine production observed following stimulation of IECs with CH-11 and TLR4 or TLR5 ligands is independent of cell death

The Fas/FasL system is best characterized in terms of its role in apoptosis, with some reports showing that cytokine expression occurs concomitantly with apoptotic cell death (25). Moreover, TLRs have been shown to induce apoptosis in certain cell types; for instance, TLR5 stimulation can induce apoptosis in IECs (20). To determine whether the augmented cytokine production observed occurred concomitantly with, or was independent of, IEC apoptosis, cells were stimulated with CH-11 and/or LPS or flagellin, and cell viability and caspase 3/7 activation were assessed. Treatment of cells with LPS, flagellin, or agonistic anti-Fas CH-11 alone or in combination did not alter cell viability in either SW480 or HT29 cells. In contrast, treatment of cells with staurosporine resulted in substantially reduced cell viability (Fig. 5A). Moreover, no increase in caspase 3/7 activity was observed in either cell line with either treatment (Fig. 5B), whereas a 4-fold induction in caspase 3/7 activity was noted in Jurkat T cells, cells known to be sensitive to Fas-mediated apoptosis, following CH-11 stimulation relative to untreated control (Fig. 5C).

Stimulation of cells with agonistic anti-Fas reduces the expression of TLR inhibitory proteins

Previous studies in macrophages have shown that in the absence of Fas signaling, the Fas adaptor protein, FADD, is present in the cytoplasm bound to the TLR adaptor protein, MyD88, suppressing TLR-induced cytokine production (14, 15). Engagement of Fas prevented this interaction, releasing MyD88 and promoting TLR-mediated inflammation. To address the potential mechanism whereby stimulation of Fas augments TLR4- and TLR5-induced TNF-α and IL-8 protein in IECs, we first investigated whether FADD interacts with MyD88. Tagged constructs of FADD and MyD88 were overexpressed in HCT116 cells, and

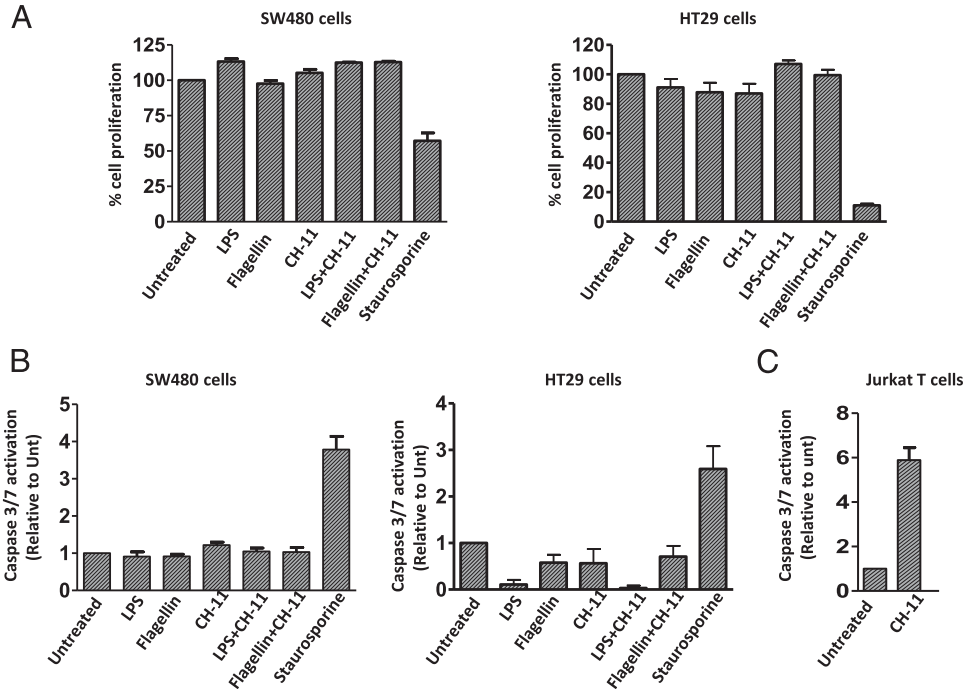

FIGURE 5. The augmented cytokine production is not dependent on caspase activation. Cells were treated with 50 ng/ml agonistic anti-Fas (CH-11) for 1 h, followed by stimulation with 100 ng/ml LPS and/or 100 ng/ml flagellin for 24 h. Cell proliferation was determined by resazurin reduction (**A**) and caspase 3/7 activation by fluorescence (**B** and **C**).

coimmunoprecipitation studies were performed. However, no interaction between FADD and MyD88 was observed in this cell type (data not shown).

TLR signaling is regulated by an extensive array of TLR inhibitory proteins such as SIGIRR and TOLLIP (26, 27). Thus, we investigated whether signaling through Fas altered the expression of TLR inhibitory proteins shown to be important in the intestine. Cells were stimulated with CH-11, LPS, and/or flagellin, and changes in a panel of TLR inhibitory proteins were assessed by RT-PCR (Table I). Stimulation of SW480 cells with CH-11 reduced the transcription of SIGIRR, TOLLIP, and 14-3-3 ε, but not IRAK-M or PPARγ (Fig. 6A). Furthermore, costimulation with CH-11 and either LPS or flagellin resulted in a greater reduction in expression of 14-3-3 ε. The change in protein levels of SIGIRR, but not IRAK-m, was confirmed by Western blotting (Fig. 6B). Together these findings suggest that the augmentation in TLR4- and TLR5-induced cytokine production by Fas may be due to its ability to downregulate the expression of several key TLR inhibitory proteins.

Suppression of Fas expression limits the ability of HT29 cells to respond to TLR4 and TLR5 ligands

Given the cross-talk seen between the Fas and TLR4 and TLR5 signaling pathways, we next sought to determine whether suppression of Fas expression altered the response of the IECs to TLR ligands. Fas was suppressed in HT29 cells, using shRNA specific against Fas ($HT29^{Fas\ shRNA}$). Suppression was confirmed by both Western blotting (Fig. 7A) and functional analysis; CH-11 did not induce IL-8 in $HT29^{Fas\ shRNA}$ cells, in contrast to the induction seen in cells transfected with scrambled shRNA ($HT29^{scr\ shRNA}$) (Fig. 7C). The specificity of suppression was confirmed by immunoblotting for TNFR-1 (Fig. 7B).

Both LPS- and flagellin-induced IL-8 production was reduced to the level of unstimulated control cells following suppression of Fas expression (Fig. 7C). This reduction was not due to any alteration in TLR4 or TLR5 expression in the knockdown cells (Supplemental Fig. 1). To determine whether IL-8 secretion in response to other TLR ligands, or known inducers of IL-8 such as TNF-α, was also affected by Fas suppression, cells were stimulated with Pam3CSK4, CpG DNA, and TNF-α. HT29 cells did not secrete IL-8 in response to Pam3CSK4, irrespective of Fas expression. However, HT29 cells did secrete IL-8 in response to stimulation with CpG DNA and TNF-α, and this was unaffected by Fas suppression (Fig. 7C).

These results were confirmed in CT26 murine IECs. Fas was stably suppressed in these cells by shRNA ($CT26^{Fas\ shRNA}$) (Fig.7D). As murine cells do not produce IL-8, the induction of CXCL-1, a murine IL-8 homolog, was determined. Consistent with suppression of Fas expression, the $CT26^{Fas\ shRNA}$ cells failed to upregulate CXCL-1 in response to Jo-2 (a murine agonistic anti-Fas Ab) (Fig. 7E). The ability of LPS to induce CXCL-1 in $CT26^{Fas\ shRNA}$ was also reduced to basal levels consistent with our findings in HT29 cells. As CT26 cells do not express TLR5 (28), we were unable to assess their response to flagellin (Fig. 7E). These findings suggest that signaling through Fas augments TLR4- and TLR5-induced cytokine and chemokine production in IECs.

Fas is required for the response of IECs to intestinal pathogens L. monocytogenes *and* S. typhimurium

Given that the IEC response to TLR4 and TLR5 ligands was attenuated upon suppression of Fas expression, the ability of $HT29^{Fas\ shRNA}$ cells to respond to the LPS-expressing Gram-negative bacterium *S.typhimurium* or the flagellin-expressing bacterium *L. monocytogenes* was assessed. In contrast to $HT29^{scr\ shRNA}$ cells, the ability of $HT29^{Fas\ shRNA}$ cells to produce IL-8 in response to stimulation with either bacterium was suppressed (Fig. 8). This finding was shown in two separately derived knockdown clones. Taken together, these findings suggest that Fas signaling is likely to play a role in intestinal host defense against pathogens.

Discussion

The aim of this study was to investigate the cross-talk between TLRs and Fas in the intestinal epithelium. Our principal findings are that stimulation through TLRs 4 and 5, either by their cognate ligands or by commensal flora, increases expression of Fas and FasL in vitro and in vivo, and that stimulation of Fas in intestinal

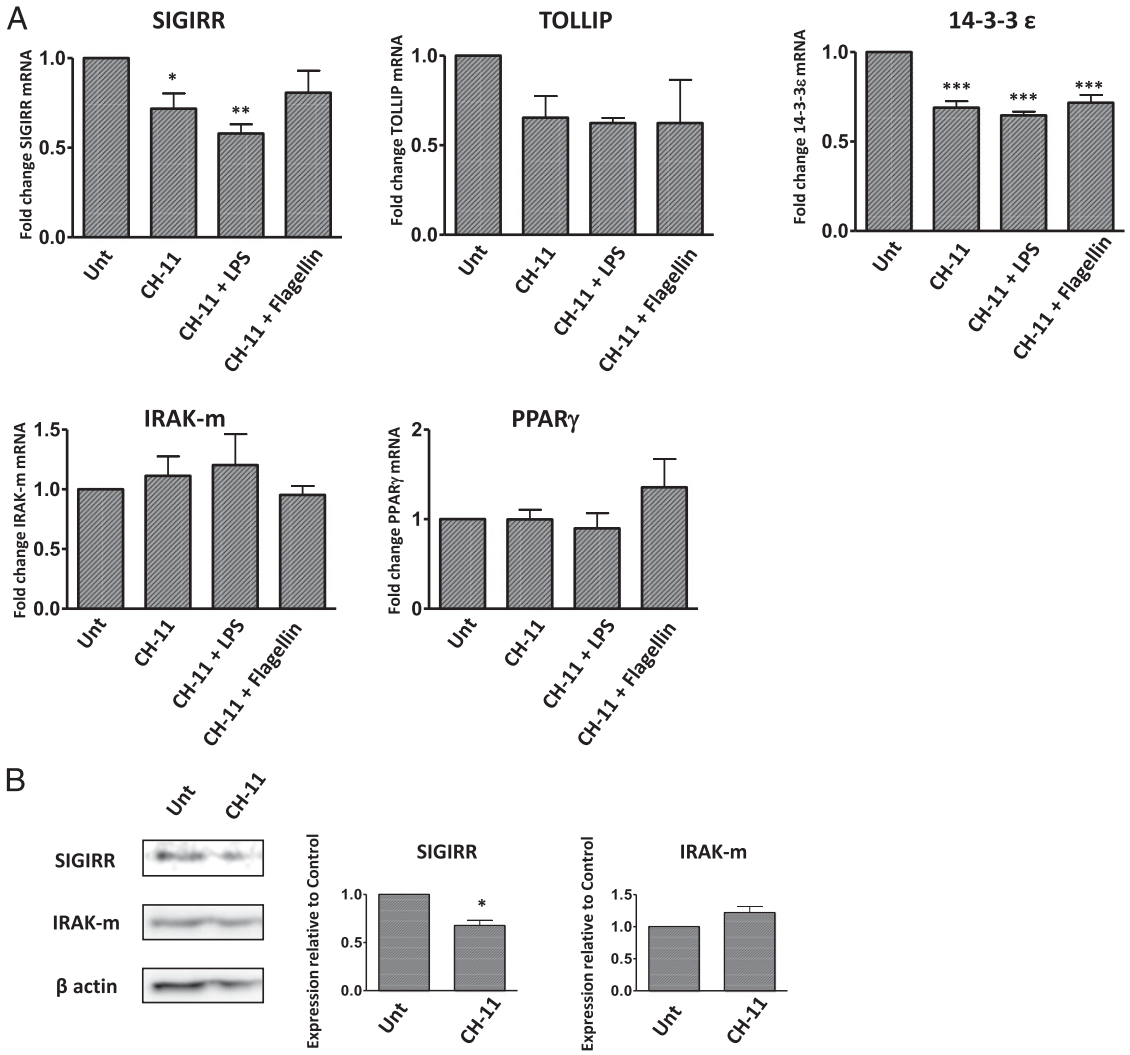

FIGURE 6. Fas activation reduces the expression of key TLR inhibitory proteins in IECs. SW480 cells were treated with 200 ng/ml agonistic anti-Fas Ab (CH-11) for 1 h followed by stimulation with 100 ng/ml LPS or 100 ng/ml flagellin for 4 h. Changes in inhibitory proteins were detected by qRT-PCR (**A**). Cells were treated with CH-11, as above, for 24 h, and changes in protein expression were detected by Western blotting and quantified by densitometry (**B**). Results shown are representative of three independent experiments. Statistical analysis was performed using one-way ANOVA (A) and the paired Student *t* test (B), and statistical change was determined compared with untreated control. Values are shown as mean ± SEM ($n = 3$).*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

cells augments TLR4- and TLR5-induced cytokine and chemokine production. These observations are significant, as they indicate a hitherto unappreciated role for Fas in the intestinal immune response.

Although upregulation of Fas by TLR2, TLR3, TLR4, TLR7, and TLR9 ligands (29–31) has been previously reported in macrophages, to the best of our knowledge no report has demonstrated an upregulation of Fas and FasL by pathogen-activated molecular patterns in IECs. In IECs, LPS and flagellin upregulated Fas and FasL expression, whereas Pam3CSK4 and CpG DNA had no effect. Moreover, we found that expression of both Fas and FasL was reduced in the epithelial cells of the colon of TLR4 and TLR5, but not TLR2, KO mice. In addition to the obvious differences in cell lineage, another possibility for the differences observed between IECs and macrophages is that upregulation of Fas and FasL expression may occur in a TRIF-dependent manner in IECs. It is well established that TLR4 uses the adaptor molecule TRIF (23), and more recently TLR5 has been shown to use TRIF in IECs (24), whereas neither TLR2 nor TLR9 has been reported to use TRIF. Of interest, a recent study examining the respective importance of TRIF-dependent versus MyD88-dependent gene expression in IECs revealed that the number of TRIF-dependent genes by far exceeded the number of genes regulated by the adaptor protein MyD88 (32). Consistent with this finding, expression of Fas and FasL was greatly reduced in TRIF KO mice, with only a modest reduction observed in MyD88 KO mice. This observation suggests that the specificity of TLR-induced upregulation of Fas and FasL in IECs may be due to the ability of TLRs 4 and 5 to use TRIF.

In this study, we found that germ-free mice have reduced Fas and FasL expression, and colonizing germ-free mice effectively "rescues" the phenotype of reduced Fas and FasL expression. This finding suggests that signaling from the commensal microbiota in IECs, via TLRs, regulates Fas and FasL expression. Recognition of commensal bacteria through TLRs also plays a role in intestinal homeostasis, as mice deficient in TLR4 or MyD88 exhibited increased morbidity and mortality with DSS-induced colitis (33). Our data indicate that TLR4 KO mice, as used in the study of Rakoff-Nahoum et al. (34), would also have reduced Fas and FasL expression. Of note, a recent study has shown that Fas can play a cytoprotective role in the intestinal epithelium (13). Thus, failure of the commensal flora to upregulate Fas and FasL in TLR4 KO

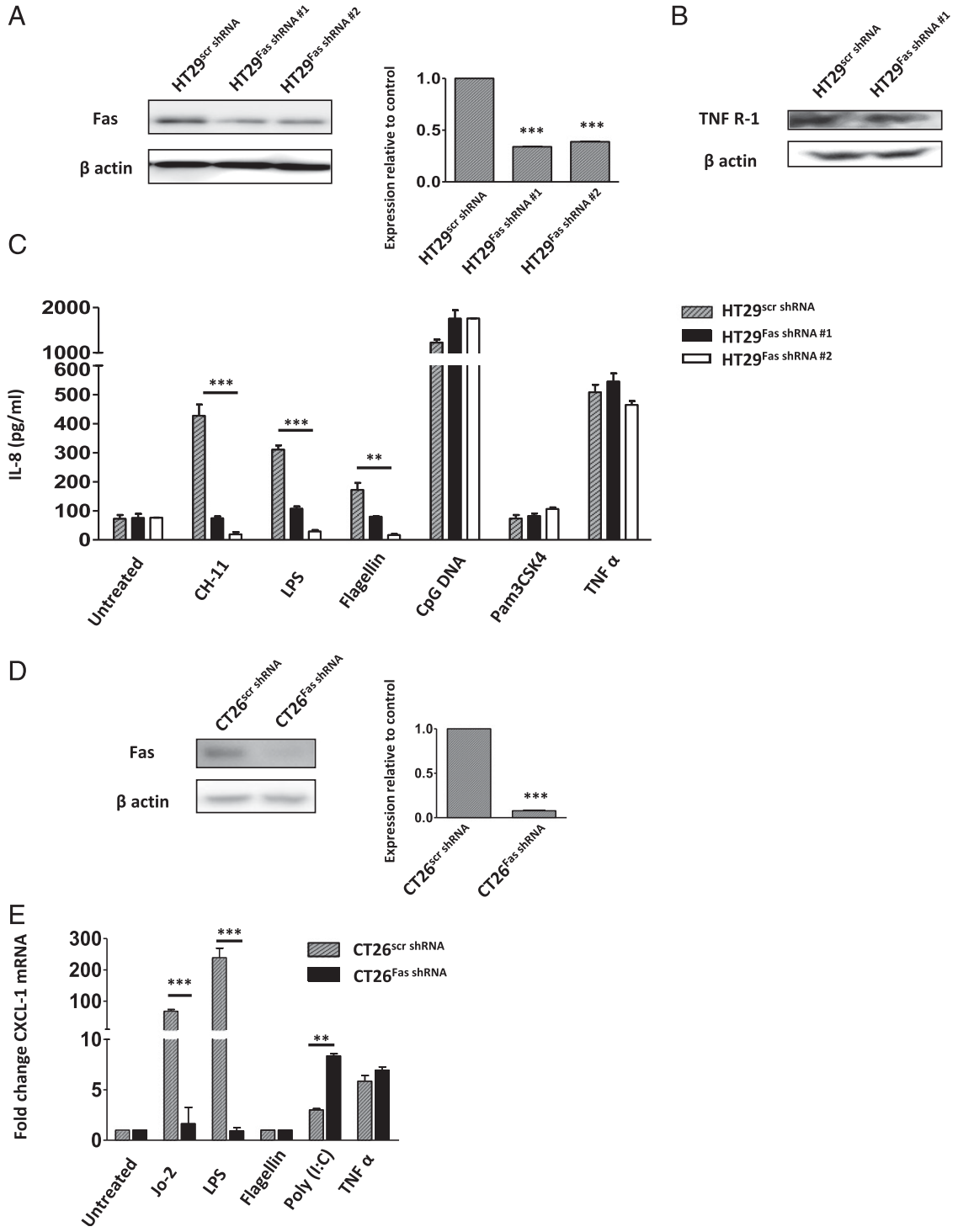

FIGURE 7. Suppression of Fas expression in HT29 cells prevents TLR4/5-induced IL-8 production. HT29 cells were lentivirally transfected with either scrambled control shRNA or shRNA against Fas. Expression levels of Fas and TNFR-1 in HT29 scrambled control transfected ($HT29^{scr\ shRNA}$) and $HT29^{Fas\ shRNA}$ clones were determined by Western blotting (**A** and **B**). $HT29^{scr\ shRNA}$ and $HT29^{Fas\ shRNA}$ clones were treated with either 200 ng/ml agonistic anti-Fas Ab (CH-11), 100 ng/ml flagellin, 100 ng/ml LPS, 5 μM CpG, 10 μM Pam3CSK4, or 100 ng/ml TNF-α for 24 h. Cell supernatants were collected, and IL-8 protein concentration was determined by ELISA (**C**). CT26 cells were lentivirally transfected as above, and expression levels of Fas were determined by Western blot (**D**). CT26 cells were treated with either 100 ng/ml agonistic anti-Fas Ab (JO-2), 100 ng/ml LPS, 100 ng/ml flagellin, 1 mg/ml polyinosinic-polycytidylic acid, or 100 ng/ml TNF-α for 4 h. Changes in mCXCL-1 were detected by qRT-PCR (**E**). Statistical analysis was performed, and statistical change was determined between the scrambled and knockdown clones. Values are shown as mean ± SEM ($n = 3$). $^{**}p < 0.01$, $^{***}p < 0.001$.

mice may exacerbate the intestinal injury seen in these mice upon DSS administration. Our data would also indicate the possibility that other, as yet unknown, proteins may also be absent in TLR4 and TLR5 KO mice, resulting in roles being ascribed to TLRs that are actually due to the absence of other proteins.

Despite being best characterized as an apoptotic inducer, activation of Fas did not alter the sensitivity of the IECs to Fas-mediated apoptosis. Fas activates the extrinsic apoptotic pathway, wherein activation of Fas induces the formation of a death-inducing signaling complex, resulting in the cleavage and activation of procaspase-8.

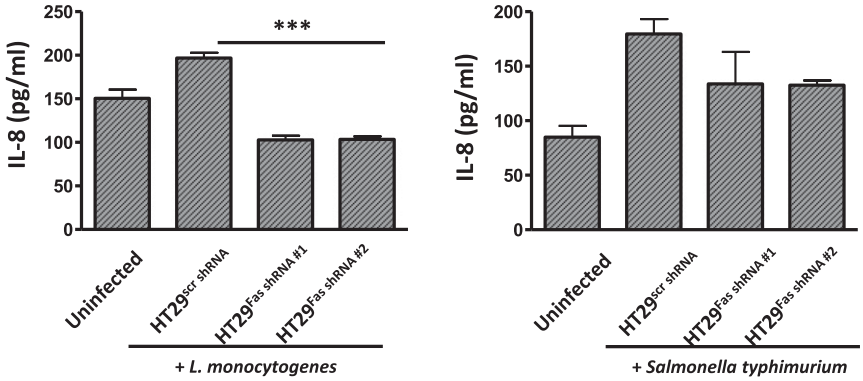

FIGURE 8. Fas is required for the induction of IL-8 in response to *L. monocytogenes* and *S. typhimurium* infection in IECs. Confluent HT-29 cells were treated with *S. typhimurium or L. monocytogenes* (50×10^7 CFU/ml) for 3 h, followed by treatment with gentamicin (50 ng/ml), and IL-8 protein levels in cell culture supernatants were measured after 24 h by ELISA. Values are shown as mean ± SEM (n = 3). Statistical analysis was performed, and statistical change was determined compared with infected scrambled control. ***$p < 0.001$.

Caspase-8, in turn, activates a caspase cascade, culminating in the apoptotic death of the cell. Numerous proteins, including c-FLIP, have been shown to suppress Fas-mediated apoptosis. IECs express high levels of c-FLIP, which suppresses Fas-mediated apoptosis through its ability to prevent processing of procaspase-8 to its mature active form (35, 36). Thus, despite coexpressing Fas and FasL, IECs are protected against Fas-mediated apoptosis under homeostatic conditions. Given that Fas has been shown to mediate several nonapoptotic functions, such as inflammation (11), our data suggest that in IECs, these nonapoptotic functions of Fas may predominate.

The findings of this study also demonstrate extensive cross-talk between the Fas and TLR signaling pathways in IECs in terms of cytokine induction and response to bacterial infection. One of the first indications of a cross-talk between the Fas and TLR signaling pathways came from studies in macrophages. LPS-activated macrophages produce a large amount of IL-1β upon FasL stimulation (14, 31), and TLR4 signaling is reduced in *lpr/lpr* and *gld/gld* peritoneal macrophages, which have mutations in Fas and FasL, respectively (37). The proposed mechanism for this cross-talk was that MyD88 was inhibited by FADD via a direct interaction between the adaptor proteins (14), and that activation of the Fas signaling pathway prevented this interaction. We were, however, unable to detect this interaction in IECs, suggesting that the cross-talk seen in IECs occurs via a different mechanism. We subsequently determined that in IECs, Fas signaling alters the expression levels of TLR inhibitory proteins. TLR signaling is tightly regulated by a range of TLR inhibitory proteins, several of which have been shown to be important in intestinal homeostasis (38, 39). Stimulation of Fas in IECs reduced the expression of three of the five inhibitory proteins examined by 20–40%. This level of reduction is in line with that seen in other studies, and has been shown to be sufficient to alleviate the suppression of TLR signaling (40). Of those reduced, both SIGIRR and Tollip have been previously shown to be essential negative TLR regulators in IECs (38, 41), with a reduction in expression of SIGIRR, in particular, being associated with exacerbated colitis in murine models (40, 42). We also observed a reduction in 14-3-3 ε, which has recently emerged as a TLR inhibitory protein (43). It is worth noting that we did not observe a reduction in CpG DNA–induced IL-8 in our HT29$^{\text{Fas shRNA}}$ knockdown clones. This finding may be attributed to the fact that the expression of several inhibitory proteins examined remained unchanged upon Fas activation and that these may be more important in suppressing TLR9-mediated inflammation in IECs. Thus the augmentation of TLR4- and TLR5-induced cytokine production seen upon Fas stimulation may be, at least in part, due to the reduced expression of 14-3-3 ε, Tollip, and SIGIRR.

In summary, we have shown that extensive cross-talk exists between Fas and TLRs 4 and 5 in IECs in vitro and in vivo. Our data further advance the evidence in favor of nonapoptotic functions of Fas and reveal a hitherto unknown link between Fas and FasL expression and the presence of commensal flora in IECs. The alterations in TLR-mediated cytokine production shown in this study, upon manipulation of both the expression and the activation of Fas, suggest that targeting Fas has potential therapeutic applications and warrants further investigation.

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Disclosures

The authors have no financial conflicts of interest.

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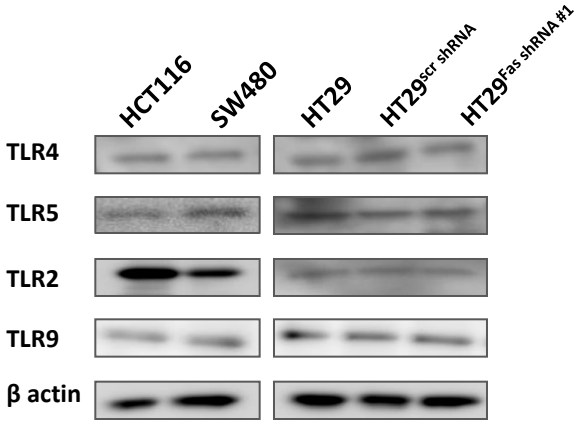
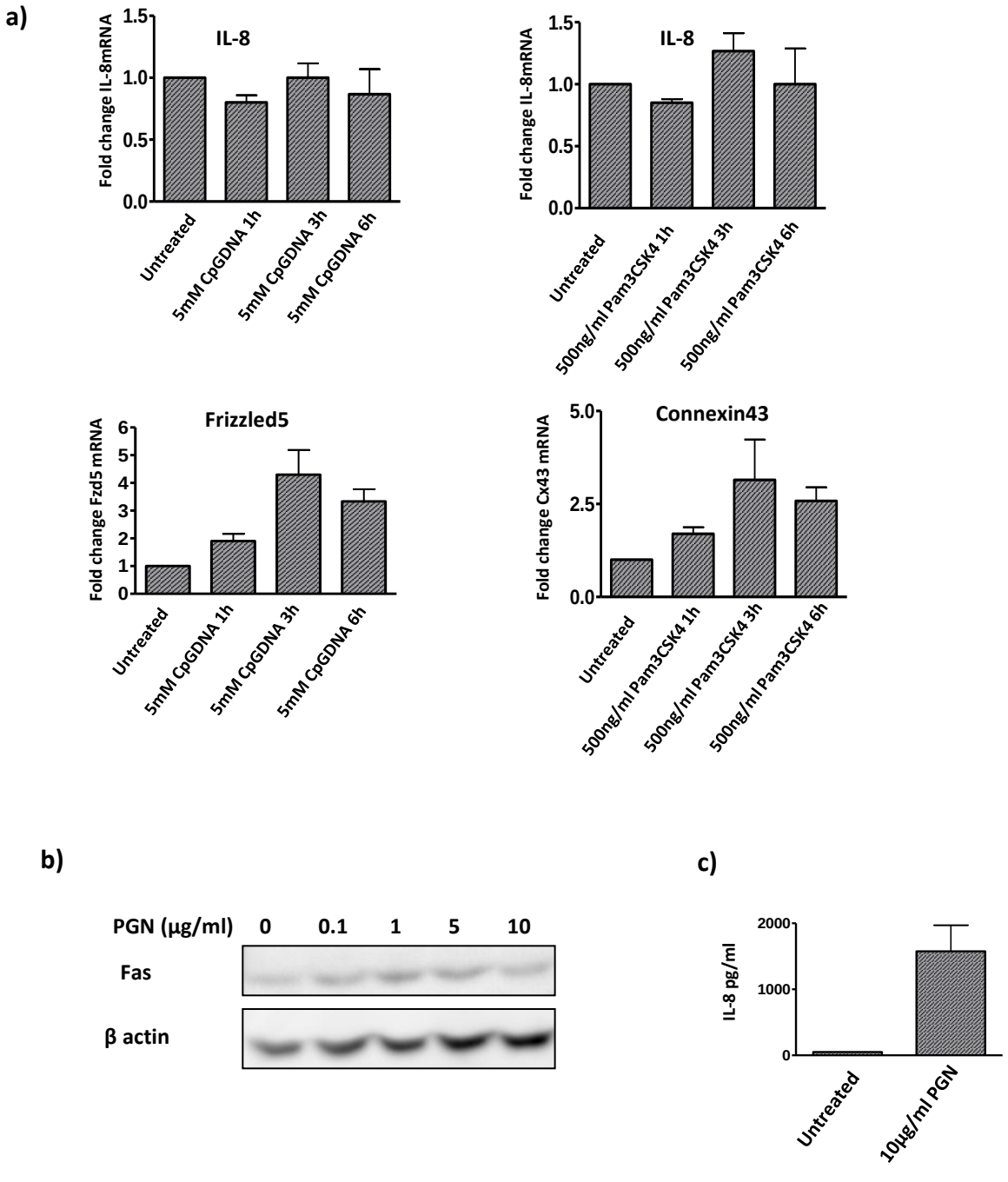
Supplementary data – S1



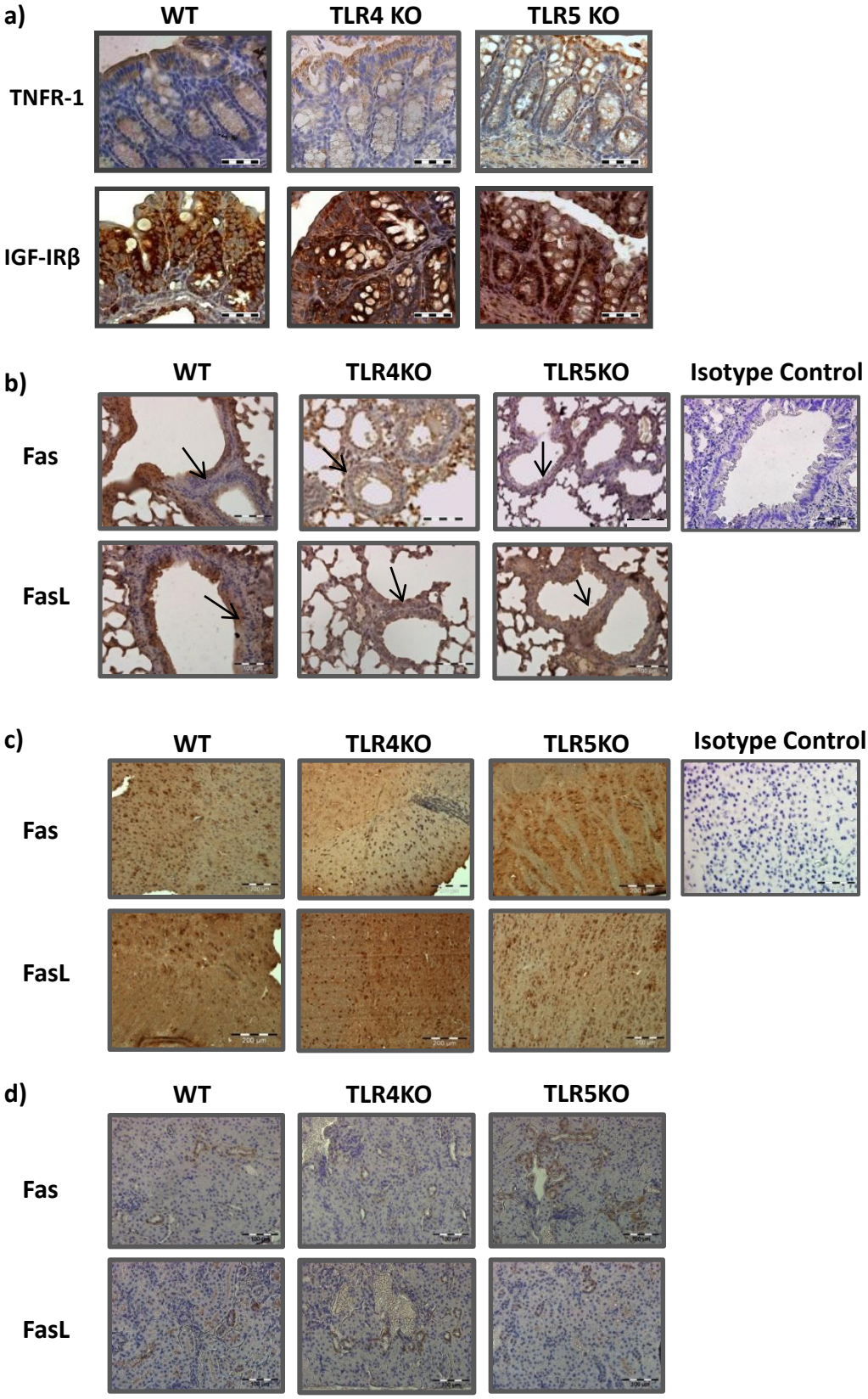
Supplementary Figure S1. Intestinal epithelial Cells express TLRs 2, 4, 5 and 9.
Cells were seeded at a concentration of 1 x 10^6 cells per ml. Protein expression was detected by Western blotting.

Supplementary data – S2



Supplementary Figure S2. SW480 cells respond to TLR2 and TLR9 agonists.
SW480 cells were seeded at a concentration of 1 x 10^5 cells per ml. Cells were treated with CpGDNA or Pam3Csk4 as specified and fold change of IL-8, Frizzled5 and Connexin43 determined by RT-PCR **(a)**. Cells were treated with PGN for 24 hours and changes in Fas detected by Western blotting **(b)** and changes in Il-8 detected by ELISA **(c).**

Supplementary data – S3



Supplementary Figure S3: **Fas and FasL expression is reduced in the lung but not the cerebellum or kidney of TLR4 and TLR5 knockout mice.**

Immunoperoxidase staining for TNFR-1, IGF-IRβ, Fas, FasL was performed on paraffin embedded **a**) colonic **b**) lung **c**) cerebellum and **d**) kidney tissue sections obtained from wildtype, TLR4KO and TLR5KO C57BL/6 mice. Scale bar = 100µM where not otherwise specified